University of Alberta

SEMEN ASSESSMENT TECHNIQUES FOR DETERMINING RELATIVE BOAR FERTILITY

by

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I dedicate this thesis to

the memory of María Macedo de Sánchez.

(1904-2006)

ABSTRACT

Semen evaluation commonly used by commercial swine AI centers detects serious problems with the quality of individual ejaculates and boar fertility, but is unable to estimate the relative fertility of individual boars used for AI. Therefore, reliable indicators of ejaculate quality that allow exclusion of low quality boars for use in AI are needed. Based on a review of current literature, the use of the *in vitro* tests that evaluate sperm characteristics directly related to the fertilization process,, analysis of seminal plasma proteins, changes in sperm motility in extended semen used for AI, and the use of low sperm numbers per AI dose were hypothesized to have potential for providing meaningful information on relative boar fertility. Based on these concepts, effective predictors of semen quality and relative boar fertility were evaluated. In the first experiment, the use of low sperm numbers for AI, pregnancy rate at Day 30, motility of extended semen at D7 and D10 of storage, and specific IVF parameters were useful for characterizing relatively infertile boars. In a second experiment, seminal plasma proteins were evaluated and fertility *in vivo* was positively correlated with a 25 kDa, pI 5.9 protein, but negatively correlated with the relative abundance of PSP-I, as well as a 20 kDa, pI 6.0 and a 60 kDa, pI 6.5 protein. In a third study, boars were selected and grouped on the basis of motility scores of extended semen at D7 and 10 of storage to investigate the relationships with boar fertility. The results confirmed that assessment of sperm motility in extended semen at D7 and 10 of storage may offer a practical and inexpensive approach for identifying less fertile boars. Collectively, these studies suggest that the use of low sperm numbers for AI and associated pregnancy rate at D30,

motility of extended semen at D7 and 10, specific IVF parameters, and specific seminal plasma proteins, may be useful for identifying relatively infertile boars that are not currently identified with conventional semen evaluation techniques.

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LIST OF ABREVIATIONS

ABP	Androgen-binding protein
AC	Acrosome reaction
AI	Artificial insemination
ALH	Amplitude of lateral sperm head displacement
ASG	Accessory sex glands
ASGC	Alberta Swine Genetics Corporation
AT	Antithrombin
BSP	Bovine seminal protein
BTS	Beltsville Thawing Solution
CHAPS	3[C3-Cholamidopropyl)dimethylammonio]-1propane-sulfonate.
CASA	Computer-assisted semen analysis
CE	Caudal epididymis
CFDA	Carboxyfluorescein diacetate
COC's	Cumulus-oocyte-complexes
d	Day
DCP	Decapacitation factors
DFI	DNA fragmentation index
DHT	Dihydrotestosterone
Di	Diploid pachytene spermatocyte
DPS	Daily sperm production per gram of testis parenchyma
D-PSP-I	Deglycosylated seminal plasma PSP-I
E	Estrogen
FPP	Fertilization promoting protein
FI	Fertility index
FMP	Forward-motility protein
FSH	Follicle stimulating hormone
g	gram
GnRH	Gonadotropin-releasing hormone
H boar	High motility boars

HCO_3^-	Bicarbonate
HDL	High-density lipoproteins
Hep^+	Heparin-binding proteins
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid]
hIVP	Homologous *in vitro* penetration assay
HOST	Hypoosmotic-swelling test
HSP	Horse seminal protein
HZA	Hemizona assay
IGF-I	Insulin-like growth factor-1
IVF	*In vitro* fertilization
IVM	I*n vitro* matured
kDa	Kilo Daltons
L	Liter
L boar	Low motility boar
LH	Luteinising hormone
LSM	Least-square means
MCP	Membrane cofactor protein
min	minute
mL	milligram
mo	month
MPN	Male pronuclei per penetrated oocyte
MPN-f	Percentage of penetrated oocytes with at least one male pronucleus
NCSU	North Carolina State University
OD	Optical density
OPN	Osteopontin
P	Probability
PCI	Protein C Inhibitor
pFF	Porcine follicular fluid
PI	Preleptotene spermatcyte
PI	Propidium iodide

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase
PS	Pachytene spermatocyte
PSP-II	Seminal plasma protein-I
PSP-II	Seminal plasma protein-II
PVA	Polyvinylalchohol
re/mL	Relative units per mL of seminal plasma
r	Correlation coefficient
r^2	Regression coefficient
ROS	Reactive oxygen species
SAS	Statistical Analysis System
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SF	Sperm-Free fraction
SMIF	Sperm motility inhibiting factor
SP	Sperm-Peak fraction
SP-1	Stallion seminal plasma protein 1
SPMI	Seminal plasma motility inhibitor
SPO	Sperm penetrated per oocyte
SQA	Sperm Quality Analyzer
SR	Sperm-Rich fraction
SRTC	Swine Research Technology Centre
SZP	Sperm penetraing the zona pellucida.
T	Testosterone
TBS	Tris buffered saline
TGF-ß	Transforming growth factor beta
UTJ	Utero-tubal junction
V	Volts
v/v	Volume per volume
VCL	Curvilinear velocity

VSL	Straight line velocity
wk	Week
w/v	Weight per volume
χ^2	*Chi*-square
Z	Zygotene spermatocyte
ZP	Zona pellucida
ZPBP	Zona pellucida binding protein
>1MPN	Percentage of penetrated oocytes with more than one male pronucleus
1MPN	Percentage of penetrated oocytes with one male pronucleus

CHAPTER ONE

INTRODUCTION

Genetic improvement in the livestock industry requires that high ranked sires generate as many offspring as possible in a short amount of time in order to disseminate desirable characteristics. At the nucleus level of the breeding pyramid, increased genetic selection pressure augments the rate of genetic improvement, but at the same time reduces the number of animals selected for breeding. At the production level in the swine industry today, the high demand for breeding stock forces nucleus herds to reduce selection pressure and utilize a large number of boars, often with less desirable genetics, to cover the market demands. This produces a lag in genetic improvement at the nucleus level that in turn increases the genetic lag at the lower production levels of the genetic pyramid. Conversely, as the number of offspring obtained per boar increases, fewer boars are needed to meet breeding targets, allowing faster genetic improvement at the nucleus level and faster dissemination of desirable characteristics throughout the swine production industry. For this reason it is necessary to develop new techniques that will increase boar productivity and accelerate genetic improvement at all levels of the breeding pyramid.

In order to address this problem, the swine industry has increased the use of artificial insemination (AI) in the last decade (Burke, 2000; Weitze, 2000), increasing the number of offspring per boar and at the same time augmenting the demand for good quality processed semen. New reproduction technologies, including post-cervical (Watson and Behan, 2002) and deep-uterine (Vázquez et al., 2005) AI, use of frozen semen (Guthrie and Welch, 1995), embryo transfer (Abeydeera, 2001), and cloning (Polajaeva, 2001) are also under investigation as techniques for improving the efficiency of genetic flow through the breeding pyramid. Nevertheless, a major limitation to increasing boar productivity has been the lack of good indicators of semen quality and relative boar fertility. It is well known that the most accurate

indication of semen quality and boar fertility is the impregnation of the female, but this is an expensive and time-consuming test. Therefore, several studies have investigated more practical laboratory tests to evaluate semen quality. Currently, in commercial AI centers, routine semen assessment generally includes the evaluation of ejaculate characteristics such as sperm concentration, morphology, viability, and motility (Gadea, 2005). Although some of these parameters are correlated with fertility (Flowers, 1997; Xu et al., 1998), they are generally only useful to detect male reproductive disorders that result in low fertility. They are not useful for predicting relative fertility in healthy boars with ejaculate quality that meets normal industry standards (>70 % motility and <30% abnormal sperm morphology) (Flowers, 1997; Alm et al., 2006), even though the reproductive efficiency of these boars may still be substantially different (Flowers, 1997; Tardif et al., 1999; Popwell and Flowers, 2004). Furthermore these differences become increasingly evident when low sperm doses (<2.5 billion sperm) are used for AI (Watson and Behan, 2002; Tardif et al., 1999). Therefore, effective predictors of relative boar fertility could prove useful in excluding less fertile boars from commercial boar studs and optimizing the use of proven high fertility boars with lower sperm numbers per AI dose. At the nucleus level this will allow for increased selection pressure by increasing the number of offspring bred per collection from high ranking boars.

Due to the complexity of the fertilization process, several sperm attributes are required for successful fertilization. These include the ability of sperm to undergo capacitation, hyperactivation and the acrosome reaction, to bind to the zona pellucida (ZP) and penetrate the oocyte. A range of sophisticated *in vitro* tests has been used to evaluate the sperm characteristics directly related to the fertilization process, including the hypo-osmotic-swelling test (Vázquez et al., 1997; Chun-Xia and Zeng-Ming, 2000; Pérez-Llano et al., 2001), sperm-ZP binding test (Fazeli et al., 1995; Berger et al., 1996), and the *in vitro* fertilization (IVF) of homologous, zona-intact, *in vitro* matured (IVM) oocytes (Xu et al., 1996a-b; Xu et al., 1998). However, only ZP penetration rate (Ivannova and Mollova, 1993) and oocyte penetration rate (Berger and Parker, 1989; Gadea et al., 1998; Xu et al., 1998; Gadea and Matas, 2000) have

been successfully used to identify fertile versus subfertile boars and/or ejaculates. Despite these results, it is unclear whether these techniques would be effective for predicting the relative fertility of boars with ejaculates that meet normal laboratory criteria for extending and use for AI.

The main obstacle to establishing relationships between characteristics of an ejaculate identified in the laboratory and proven boar fertility may be the use of high sperm doses for AI, and also doses comprising pooled semen collected from two or more boars, which tends to compensate for differences in fertility among boars. The importance of using lower sperm doses for AI to determine relative boar fertility *in vivo* has been confirmed in a number of subsequent studies (Tardif et al., 1999; Watson and Behan, 2002; Ardón et al., 2003). This approach likely avoids the compensatory effect of using excessive sperm numbers per AI dose (Saacke et al., 2000; Alm et al., 2006), allowing the detection of fertility differences between relatively fertile boars.

In order to find a truly effective method of assessing semen quality, it is necessary to understand the different steps in the fertilization process, including sperm formation, characteristics and functions, sperm transportation, sperm storage in the female tract, and all the changes that the spermatozoon undergoes in preparation for its fusion with the oocyte. A major problem is that many steps of the fertilization process are poorly understood, thus development of an effective test to predict male fertility is difficult. Nevertheless, with new advances in the technologies related to this field, these goals are becoming more achievable, and the development of such discriminatory tests is the subject of the studies presented in this thesis.

The information presented in this Introductory section is followed in Chapter 2 by a discussion of the general background biology of spermatozoa and seminal plasma, with a specific focus on information linking sperm characteristics with sperm function. An overview of the events occurring before and during fertilization is also presented. Finally, information on existing tests used to assess semen quality is

presented. The understanding gained from this review is then applied to identifying the most effective test or tests to assess semen quality and boar fertility.

As a part of the research component of this PhD program, three studies were conducted and are presented in sequence in Chapters (3, 4 and 5). Chapter 3 presents the first study that evaluated potential indicators of relative boar fertility using a population of healthy boars with ejaculate quality that met normal industry standards (>70 % motility and <30% abnormal sperm morphology). Semen from these boars was used to breed gilts with low sperm AI doses for *in vivo* fertility evaluation (1.5 billion sperm per AI dose). In addition, a standardized IVM-IVF technique, previously used in our laboratory (Xu et al., 1998), was employed to assess boar fertility *in vitro*. Multiple regression models were used to evaluate the relationship between IVF parameters, routine semen evaluations results and relative boar fertility determined *in vivo*. The use of low sperm numbers for AI, pregnancy rate at d30, motility of extended semen after 7 and 10 days, and specific IVF parameters appear to be useful for identifying relatively infertile boars that are not currently excluded from use in existing commercial boar studs.

As an extension of the first study, associations between seminal plasma proteins and observed fertility were examined (Chapter 4). Total protein concentration analysis, immunoblotting techniques to quantify porcine specific proteins (Seminal Plasma Protein-I (PSP-I), AWN-1 and osteopontin), and 2-D gel electrophoresis were used. Differences in total protein and specific proteins concentrations between fractions were observed. Fertility *in vivo* was positively correlated with a 25 kDa, pI 5.9 protein, but negatively correlated with the relative abundance of PSP-I, as well as a 20 kDa, pI 6.0 and a 60 kDa, pI 6.5 protein. These results suggest that, as in other domestic farm species, there are seminal plasma proteins that have specific effects on fertilization and their potential use as markers of semen quality and boar fertility merits further study.

A third study described in Chapter 5 was carried out in collaboration with

Alberta Swine Genetics Corporation (ASGC) and further evaluated sperm motility in stored extended semen as a predictor of boar fertility. This provided the opportunity to evaluate a larger number of commercial AI boars during their training period. Boars were selected and grouped based on the motility scores of extended semen at Days 7 and 10 of storage to investigate the relationship between this parameter and boar fertility. These data suggest that assessment of sperm motility of extended semen at Days 7 and 10 of storage may offer a practical and inexpensive approach for identifying less fertile boars. This presents an excellent opportunity for use at a commercial level that could have considerable economic impact on the productivity of the swine industry.

A general discussion of these studies and their implications is presented in Chapter 6 of this thesis. The Appendix contains the results of various pilot studies conducted in relation to the main studies presented, including: 1) *In vitro* evaluation of the fertility of different ejaculate fractions (Sperm-Peak fraction versus Sperm-Rich fraction); 2) Optimization of centrifugation rate for evaluation of seminal plasma proteins; 3) Boar seminal plasma effects on AI outcomes; 4) Evaluation of different techniques for determining sperm concentration (haemocytometer versus colorimeter).

Collectively, the studies presented in this thesis suggest that the use of low sperm numbers for AI, determination of pregnancy rate at d30, motility of extended semen after 7 and 10 days, specific IVF parameters, and specific seminal plasma proteins, may be useful for identifying relatively infertile boars that are not currently excluded from use in existing commercial boar studs.

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CHAPTER TWO

LITERATURE REVIEW

This chapter provides an overview of spermatozoa structure, origin, development and function, as well as the origin, composition and function of seminal plasma. This background provides the fundamental basis for discussing the different semen assessment techniques used to estimate male fertility.

2.1. THE SPERMATOZOON STRUCTURE, ORIGIN, DEVELOPMENT AND FUNCTION

The male reproductive system produces and transports the spermatozoa to the female tract for fertilization. The male reproductive tract includes the testicles, vas efferens, epididymis, vas deferens, urethra, accessory sex glands (seminal vesicles, ampulla, bulbourethral glands, prostate) and penis (Figure 2-1). In general, semen consists of two major components, spermatozoa and seminal plasma, each with unique origins, compositions and functions. While spermatozoa are produced within the seminiferous tubules of the testes, seminal plasma is secreted by the epididymis and the accessory sex glands. An understanding of spermatozoa structure and origin, and the events that occur from their formation in the testes up until the point of fertilization in the female tract will be provided in the first part of the literature review.

2.1.1. Morphological features of the spermatozoon

The size and shape of the spermatozoa differ among mammalian species. However, the common and principal components of the spermatozoa are the head and tail enclosed by a single plasma membrane (Figure 2-2).

The sperm chromosomal material, tightly packed with nucleoproteins and surrounded by a nuclear envelope, makes up about 65% of the sperm head. The acrosome, derived from the Golgi apparatus during spermiogenesis, is located over the anterior end of the sperm nucleus, and contains hydrolytic enzymes (acrosin, hyaluronidase, zona lysin, esterases and acid hydrolases) that are required for the penetration of the zona pellucida (Eddy and O'Brien, 1994 and references therein; Senger, 1999; Toshimori and Ito, 2003; Yoshinaga and Toshimori, 2003). Moreno et al. (2006) found that the microtubule organization between the Golgi apparatus and acrosome participates in acrosome formation during spermiogenesis and plays a key role in the allocation of specific acrosome proteins in round spermatids.

The tail provides energy and motility to the sperm (Woolley, 1979). The sperm tail consists of an axoneme complex, which is formed by two central single microtubules surrounded by nine double microtubules. These microtubules are capable of sliding along one another, producing tail motion (Vernon and Woolley, 2004). The mammalian spermatozoon has a layer of nine dense fibres (outer dense fibres) surrounding the axoneme complex that stiffens the sperm tail and may be involved in flagella flexibility (Linck, 1979; Miller et al., 2001; Tashibana et al., 2005). In general, the tail is divided into four segments: the connecting piece or neck, the middle piece, the principal piece and the terminal piece. The neck, is a short constricted segment that joins the sperm head to the tail and its flexibility allows the sperm tail to move from side to side to facilitate sperm motility. The middle piece around the axoneme complex contains elongated highly specialized mitochondria, which produces the energy substrate (ATP) for the activity of the contractile fibres of the tail. In mammals the principal piece also possesses a cytoskeletal structure, called the fibrous sheath, which may be responsible for regulation of the sperm tail rhythm (Eddy and Brien, 1994 and references therein; Senger, 1999) (Figure 2-1).

2.1.2. Spermatozoon origin

Spermatozoa are produced in the seminiferous tubules of the testes through the process of spermatogenesis. Spermatogenesis is under endocrine control and is

regulated by the hypothalamic-hypophyseal-testicular axis as illustrated in Figure 2-3. Further explanation of hormonal regulation of testicular function will be made later in this Chapter.

Spermatogenesis begins with the mitotic division of the spermatogonia, also know as stem cells (2n), located along the basement membrane of the seminiferous tubules. This mitotic division results in the formation of primary spermatocytes (2n). Primary spermatocytes undergo the first meiotic division involving cell replication and exchange of genetic material from the homologous chromosomes, which helps to guarantee genetic diversity. The first meiotic division produces two secondary spermatocytes (2n), which each divide (second meiotic division) to form two haploid spermatids (1n). Spermatids then differentiate into spermatozoa through the process of spermiogenesis. During spermiogenesis, the spermatid undergoes a series of morphological changes to become a spermatozoon. These changes include a reduction in nucleus size (chromatin condensation), loss of most of the cytoplasm, and formation of the acrosome and tail (Figure 2-4).

Spermatogonia, the most immature spermatogenic cells, lie on the basement membrane of the seminiferous tubules and as spermatogenesis progresses the more mature spermatogenic cells migrate towards the luminal region of the seminiferous tubules (Johnson, 1995; Johnson et al., 2000; Lingappa and Farey, 2000; Porterfield, 2001) (Figure 2-5). The production and release of spermatozoa along the seminiferous tubules is a continual process and different stages of spermatogenic cells can be found within segments of the tubules at any given time. However, examination of a cross-section of the tubules reveals that a complete progression of the various cellular stages is present at any given time (Senger, 1999).

Spermatogenesis spans the period from spermatogonia development and differentiation at the basal compartments, to the spermatozoa differentiation in the luminal section of the seminiferous tubules. During spermatogenesis the germ cells undergo a progressive series of changes, called stages, which are grouped in cycles as

illustrated in Figure 2-6 (Ravidindranath et al., 2003). The cycle length and total duration of spermatogenesis differs between species (Table 2-1). For example, a cycle in boars occurs over 8.6 to 9.0 days and the total duration of spermatogenesis encompassing 4.5 spermatogenic cycles is approximately 39 to 41 days. In humans, each cycle lasts 16 days and the total duration of spermatogenesis is 72 days (16 days x 4.5 cycles) (França et al., 2005; Zeng et al., 2006). Germ cells exhibit a degree of cell degeneration that varies between species, as well as the time when this degeneration occurs, affecting spermatogenic efficiency as represented by daily spermatozoa production per gram of testis parenchyma in Table 2-1 (França et al., 2005).

In the lumen of the seminiferous tubules, sustentacular or Sertoli cells are present to support, protect and nourish the developing spermatogenic cells. Sertoli cells are considered to regulate the effects of testosterone during spermiogenesis, maintaining androgen levels through the synthesis of androgen-binding protein and thus maintaining higher levels of androgens within the testes. Sertoli cells also secrete the hormone inhibin that controls the release of follicle stimulating hormone (FSH) from the anterior lobe of the hypophysis (as reviewed by Griswold and McLean, 2006; O'Donnell et al., 2006). The Sertoli cells, positioned on the basement of the tubules, also play a role in the blood-testis barrier, preventing any immune response against the spermatid cells (Wong and Cheng, 2005). Furthermore, Sertoli cells absorb the excess spermatid cytoplasm and secrete the fluid necessary to transport the sperm into the epididymis. This fluid also contains nutrients and decapacitation and motility inhibitors which preserve the viability of the spermatozoa (as reviewed by Griswold and McLean, 2006; O'Donnell et al., 2006; Kerr et al., 2006). The Sertoli cells are also responsible for phagocytosis of degenerated germ cells (Porterfield, 2001; França et al., 2005). Sertoli cell numbers are positively correlated with daily spermatozoa production in the boar (Okwun et al., 1996) and stallion (Johnson et al., 1994), and elevated numbers of type A spermatogonia and Sertoli cells are also associated with large testes and the number of spermatozoa produced in mature boars (Okwun et al., 1996; França, 2000; França et al., 2005).

Groups of interstitial cells, called Leydig cells, are located around the seminiferous tubules (Figure 2-5). As shown in Figure 2-3, Leydig cells are responsible for androgen synthesis (testosterone) and also production of estrogens, progesterone, 17α-hydroxyprogesterone and ß-endorphin, (Mendis-Handagama and Siril-Ariyaratne, 2005), insulin-like growth factor-1 (IGF-I) and transforming growth factor beta (TGF-ß) (Le Roy et al., 1999). Testosterone is mainly produced by the Leydig cells and circulating testosterone concentrations are correlated with the number of Leydig cells present. As a part of the functional blood-testis barrier, macrophages located in the interstitial space play an important role in immune activation through cytokine effects which maintain immunotolerance in the testis (Yee and Hutson, 1983; Hutson, 2006). Furthermore, interstitial macrophages couple with Leydig cells through cytoplasmic inter-digitations (Hutson, 1992) and have a paracrine interaction that seems to stimulate steroidogenesis (Yee and Hutson, 1983; Huleihel and Lunenfeld, 2004; Hutson, 2006). This occurs through the synthesis of a 25-hydroxycholesterol substrate that enables the Leydig cells to produce testosterone while skipping the STAR-cholesterol pathway (Hutson, 1992; Lukyanenko et al., 2001).

2.1.2.1. Hormonal regulation of testicular function

General hormonal regulation of testicular function in mammals has been extensively reviewed by O'Donnell et al. (2006). Spermatogenesis is under endocrine/ paracrine regulation by hormones produced by the hypothalamus (Gonadotropin-releasing hormone (GnRH)), the anterior lobe of the pituitary (Luteinising hormone (LH), Follicle-stimulating hormone (FSH)), and the Sertoli cells (dihydrotestosterone (DHT), estrogens, inhibin, androgen-binding protein) and Leydig cells (testosterone) of the testes (Figure 2-3). GnRH is released by the hypothalamus, in a frequent and intermittent manner, stimulating the release of LH and FSH from the anterior part of the pituitary. LH binds to LH receptors located in Leydig cells (testes interstitial cells), stimulating steroidogenesis (testosterone). Testosterone passes to the Sertoli cells where it is transformed into dihydrotestosterone (DHT) and estrogens (E).

Testosterone and estrogens also travel via the circulatory system and inhibit GnRH release from the hypothalamus and, through the indirect block to GnRH action on the pituitary, indirectly inhibit LH secretion. FSH acts directly in Sertoli cells, and in conjunction with androgens, stimulates the production of androgen-binding protein that contributes to the maintenance of the elevated concentrations of androgens required for spermatogenesis and facilitates the transport and location of testosterone within the Sertoli cells. Sertoli cells release inhibin that acts directly on the anterior lobe of the pituitary to inhibit FSH secretion. In Sertoli cells, FSH and testosterone cooperate to regulate several steps of spermatogenesis. Specifically, testosterone is involved in the control of the spermatid elongation process; as well as the maintenance of spermatogonia and the spermatocyte population by controlling sperm degeneration. Meanwhile, FSH participates in the proliferation of spermatogonial cells and the maturation of spermatocytes (McLachlan et al., 1996; Porterfield., 2001; O'Donnell et al., 2006).

Testosterone is present in seminal plasma, where its concentration may be indicative of fertility issues that could affect semen quality. Seminal plasma testosterone values have been found to be low in samples from human patients with abnormal sperm characteristics (Zalata et al., 1995) and from patients with sperm concentrations lower than 10 x 10^6/mL (Micic and Dotlic, 1983) or azoospermia (Mladenovic et al., 1993). Zalata et al. (1995) reported low concentrations of testosterone in seminal plasma samples from patients with abnormal sperm characteristics, but no correlation was found between semen quality and serum concentrations of testosterone, LH or FSH. However, the concentrations of testosterone in seminal plasma were weakly correlated with sperm concentration, sperm motility (percentage of forward motility) and ejaculate volume in boars (Peter et al., 1980) and humans (Zalata et al., 1995). Despite this, testosterone concentrations in seminal plasma are related to testicular activity, accessory sex glands function and is needed for the expression of secondary sex characteristics. Therefore, relative testosterone values in seminal plasma, rather than circulation levels, appear to be useful as a complementary test of male fertility.

2.1.3. Spermatozoa Maturation

Spermiation occurs when Sertoli cells release the spermatozoa into the lumen of seminiferous tubules (Senger, 1999) and they are transported to the rete testis passing through the efferent ductules to be collected in the caput ducts of the epididymis. When spermatozoa leave the seminiferous tubules and enter the epididymis they are immature and have a limited ability to fertilize (Hoppe, 1975; Cummins, 1976).

Spermatozoa maturation is a progressive process during which the spermatozoa undergo a series of morphological and biochemical changes during their transit through the different segments of the epididymis (caput, corpus and cauda) to become capable of fertilization in the female reproductive tract (Briz et al., 1995; Byrd, 1998). During epididymal maturation the spermatozoa: 1) develop active and progressive motility (Gaddum, 1968; Yanagimachi, 1994 and references therein; Acott et al., 1978; Acott et al., 1983), 2) lose their cytoplasmic droplet (Bloom and Nicander, 1961; Hafez and Garner, 1996; Briz et al., 1995), 3) develop nuclear chromatin stability (Sivashanmugam and Rajalakshmi, 1997), and 4) undergo a series of stage-specific morphological changes to the acrosome that are essential for the fertilization process (Tezon et al., 1985; Yoshinaga and Toshimori, 2003; Sivashanmugam and Rajalakshmi, 1997; Dacheux et al., 2005).

2.1.3.1. Spermatozoa motility maturation

Several studies have demonstrated that spermatozoa from the caput region of the epididymis demonstrate a circular motility that transforms to a forward movement as the sperm progresses through different regions of the epididymis. Gaddum (1968) demonstrated that rabbit spermatozoa exhibit characteristic patterns of motility in specific parts of the male reproductive tract, having weak vibratory movements in the seminiferous tubules and deferent ducts, increasing to circular motility in the caput epididymis, and gradually developing forward motility as they move from the corpus and then pass through the caudal region of the epididymis. Similar motility patterns

were also found in bull spermatozoa from the caput and caudal epididymal regions (Acott et al., 1983). Related to the subject of spermatozoa motility maturation, Brandt et al. (1978) working with bull spermatozoa, identified a Forward-Motility Protein (FMP) of epididymal origin. Further investigations determined that this protein is not species-specific, as it showed cross reactivity with monkey, man, dog, cat, guinea pig, rabbit, bear, and elephant sperm (Acott et al., 1979). FMP, in association with increases in intracytoplasmic levels of cyclic AMP, is considered to play an important role in the development of spermatozoa forward motility (Brandt et al., 1978; Acott et al., 1979; Acott and Hoskins, 1981; Acott et al., 1983).

The caudal epididymis works as a sperm storage compartment where sperm motility is inhibited by the caudal epididymis (CE) fluid through mechanisms that differ between species. This includes low pH of the CE fluid in bulls and dogs, and the high viscoelasticity of the CE fluid (mucin-like glycoprotein called immobilin) in rat and hamster (Carr et al., 1985).

2.1.3.2. Loss of cytoplasmic droplets

During the last stage of spermiogenesis after the elongation period, the spermatid discards most of the cytoplasm and just a small amount of residual cytoplasm, called the cytoplasmic droplet, binds to the neck region of the testicular and epididymal (caput) spermatozoa (Kaplan et al., 1984; Toshimori, 1993). The cytoplasmic droplet progressively migrates from the proximal position, near the sperm head, to a distal position of the midpiece, where it eventually detaches from the sperm tail. The process of cytoplasmic loss occurs during the transit through the epididymis sections. This aspect of sperm maturation was demonstrated by an experiment carried out by Briz et al. (1995), who evaluated sperm quality from the three different regions of the boar epididymis: In the caput epididymis, 48.7±4.9% of spermatozoa had proximal cytoplasmic droplets and just 0.3% had distal droplets. A considerable reduction of proximal droplets (0.3±0.6 %) and an increase of sperm with distal droplets (57.7±4.9 %) were found in the corpus epididymis. Finally, in the cauda epididymal region, no proximal droplets were detected and just a small percentage of

distal cytoplasmic droplets were seen (11.8±3.2%). This work confirmed that sperm cytoplasmic droplet migration takes place during epididymal transit. As well, this experiment demonstrated that the percentage of mature spermatozoa increases from the caput (48.7±4.9%) to the cauda (84.3±3.6%) epididymal regions.

Other studies have demonstrated that ejaculates with a high percentage of proximal cytoplasmic droplets have compromised fertility (Amann et al., 2000; Thundathil et al., 2001). Thundathil et al. (2001) using *in vitro* fertilization (IVF) and bull semen with a high percentage of proximal cytoplasmic droplets, demonstrated that spermatozoa with droplets did not bind to the zona pellucida. However, apparently normal spermatozoa from these ejaculates (i.e. without cytoplasmic droplets) that were able to fertilize oocytes, resulted in embryos that did not develop beyond cleavage. This suggested that these spermatozoa were functionally immature or incapable of initiating normal embryo development (Thundathil et al., 2001) probably due to incomplete DNA maturation (incomplete development of nuclear chromatin stability). These studies also confirmed previous results that found negative correlations ($r=-0.49$; $P<0.01$) between the percentage of proximal droplets and percentage of cleaved oocytes (Amann et al., 2000). Collectively, this information indicates that the absence of spermatozoa cytoplasmic droplets is not 100% proof that sperm are completely mature and that sperm maturation is a highly complex process that must be fully completed to result in a successfully fertilization process.

2.1.3.3. Spermatozoa membrane maturation

Sperm acrosome remodeling also occurs in the epididymis where proteins, glycoproteins (Tezon et al., 1985; Amann, 1987 as cited by Setchell, 1993; Hing-Sing, 1994 and references therein; Rutllant and Meyers, 2001) and lipids (Sivashanmugam and Rajalakshmi, 1997) of the sperm plasma membrane are lost, altered, or replaced in a stage-specific manner during their transit through the epididymis, together contributing to the final acrosome organization. As demonstrated in monkey spermatozoa (Sivashanmugam and Rajalakshmi, 1997), lipid organization of the

plasma membrane changes during sperm maturation, as does the formation of disulfide linkages of the nuclear chromatin structure (nuclear condensation).

The epididymal fluid undergoes changes to its chemical composition in different segments of the epididymis, and these changes may play an essential role in sperm maturation (Hing-Sing, 1994 and references therein; Syntin et al., 1996; Dacheux et al., 2005). Tezon et al. (1985) found that the spermatozoa may have epididymal proteins added to their surface during transit through the epididymis. This phenomenon was revealed by Syntin et al. (1996) while working with the boar epididymis, who confirmed that the synthesis and secretion of proteins by the epididymal epithelium differs according to epididymal region and found that several of these proteins bind to the sperm membrane in a stage-specific manner.

In summary, during epididymal maturation the sperm plasma membrane undergoes several physical (cytoplasmic droplet loss and DNA condensation) and chemical changes to its lipid and protein composition. Proteins of the plasma membrane change their location (Rutllant and Meyers, 2001), and may be altered, while others are completely replaced by new proteins. These changes to spermatozoa during their trek through the epididymis serve to: 1) prevent premature capacitation and hyperactivation, as discussed in the next section; 2) facilitate the acquisition of forward motility (Gaddum, 1968); 3) give the sperm the ability to perform fertilization-related functions, such as binding to the zona pellucida, oocyte penetration, and to undergo the acrosome reaction (Yanagimachi, 1994 and references therein; Yoshimara and Toshimori, 2003; França et al., 2005), and 4) allow for nuclear maturation (condensed DNA) that could be a requirement for normal embryo development (Sivashanmugam and Rajalakshmi, 1997; Thundathil et al., 2001).

2.1.4. Sperm capacitation

Even though mature spermatozoa have fertilization potential, they are not capable of fertilizing an oocyte until they undergo a series of membrane changes before their interaction with the oocyte. In essence, ejaculated spermatozoa are

surrounded by a protective coat that helps them to survive the journey from the epididymis to the site of fertilization in the female tract. Therefore, in order to bind to an oocyte the sperm has to remove the protective coat and expose the receptors that recognize the oocyte zona receptors and initiate the zona pellucida binding process.

Ejaculated spermatozoa therefore undergo capacitation, a process in which the spermatozoon experiences several changes in its metabolism as well as in its acrosome and plasma membranes, which are produced by variations in: membrane fluidity, intracellular ion concentrations, pH, cAMP concentration and the concentration of reactive oxygen species that make the spermatozoa competent for fertilization. This series of events allows mature spermatozoa to exhibit hyperactive motility, zona pellucida binding, undergo the acrosome reaction, penetrate the zona pellucida and finally fertilize an oocyte (Austin, 1952 as cited by Yanagimachi, 1988; Wassarman, 1999ab). Among different species, sperm capacitation occurs at different sites in the female reproductive tract (reviewed by Hing-Sing, 1994). For example, in pigs and many rodents, the oviduct is the principal site of sperm capacitation. However, in humans, capacitation is considered to start in the cervix (Hunter, 1984; Gould et al., 1985 as cited by Yanagimachi, 1994). Furthermore, the length of time for spermatozoa to capacitate *in vivo* differs among species (Davis, 1981), as well as between individuals of the same species (Petrunkina et al., 2005a). Spermatozoa capacitate at different rates due to differences in sperm subpopulations in the ejaculate. This characteristic could reflect individual fertility differences that may be indicative of an individual's reproductive potential.

During the capacitation process, spermatozoa lose decapacitation factors and undergo membrane lipid rearrangements, including a lateral redistribution of lipids, transverse redistribution of phospholipids across the lipid bilayers, and alterations in lipid metabolism (Wolf, 1996 and references therein; Wassarman, 1999ab). While the mechanisms of capacitation are poorly understood, heparin and high-density lipoproteins (HDL) seem to play an important role during this process in bull semen (Lane et al., 1999). It has been proposed that heparin or HDL bind to specific sperm

membrane proteins (decapacitation factors like bovine seminal plasma proteins), producing a cholesterol efflux. As well, in species such as the human and rat, serum albumin (Dow and Bavister, 1989) present in the female tract appears to be involved in cholesterol removal from the sperm plasma membrane. The efflux of cholesterol produces a reduction in the cholesterol/phospholipids ratio (Davis, 1981) that is responsible for changes in the membrane permeability. These membrane alterations induce the following changes: 1) changes in ion permeability that allows for an influx of Ca^{2+} (Lane et al., 1999; Parrish and First, 1993; Breitbart, 2003), HCO_3^- (Baldi et al., 1996), Na^+, CL^- and Zn^+ and efflux of K^+ (Baldi et al., 1996; Thundathil et al., 2006); 2) a rise in intracellular pH (Fraire-Zamora and González-Martínez, 2004); 3) production of reactive oxygen species (ROS) which activate the pathway of tyrosine phosphorylation (Ford, 2004); 4) plasma membrane hyperpolarization (Darszon et al., 2006); 5) remodeling of sperm plasma membrane phospholipids by the activation of protein phosphorylation through various pathways involving tyrosine kinase receptors, non-tyrosine kinase receptors, G-protein coupled receptors and cAMP/Protein kinase A (Gadella and Harrison, 2000). Protein tyrosine phosphorylation is the most important dependent pathway in this process as it is responsible for the plasma membrane phospholipid changes occurring during capacitation (Naz and Rajesh, 2004; Bailey et al., 2005). Following these alterations, the sperm plasma membrane acquires greater fluidity and sperm become more sensitive to the factors that induce the acrosome reaction (Parrish and First, 1993; Visconti and Kopf, 1998), and the sperm receptors for zona pellucida initial binding are also exposed. However, the mechanism of sperm capacitation requires further investigation.

During capacitation there is addition and removal of different sperm membrane glycoproteins that are presumably involved in the sperm acrosome reaction and zona pellucida binding events (Jimenez et al., 2002, 2003). Using lectins to detect carbohydrate residues on the sperm membrane, Jimenez et al. (2003) observed a decrease in fucose and N-acetylglucosamine and/or sialic acid residues, while mannose residues increased after capacitation in boar sperm. However, there are differences in the amount of sugar residues found in fresh, capacitated and acrosome

reacted ejaculates from fertile and subfertile boars. From a practical perspective these differences could potentially be used as indicators of male fertility. Furthermore, in a recent study (Petrunkina et al., 2005a), the ability of sperm to respond to stimuli that induce capacitation was evaluated and showed intra-boar variation of about 30%. Differences between fertile and subfertile boars were also detected, with subfertile boars exhibiting an extreme capacitation response and classified as high and low responders, while fertile boars presented an intermediate response to sperm capacitation stimuli. These data again allow the conclusion that the evaluation of such differences could be used as a complementary test for assessing boar fertility.

2.1.5. Sperm hyperactivation

Sperm can undergo changes in motility referred to as hyperactivation. It is now known that hyperactivation occurs independently of both sperm capacitation and the acrosome reaction processes (Márquez and Suarez, 2004). During hyperactivation, the beating of the sperm tail starts at the neck and propagates through the middle, principal and the end piece of the tail, (Ho and Suarez, 2001), flagellar amplitude increases, and the linearity of the sperm swimming trajectory changes to a circular form (Wolf and Lanzendorf, 1991 as cited by Zaneveld, 1996; Nichol et al., 1997). Recent studies demonstrated that while non-hyperactivated sperm exhibited an increase in beat frequency and a small change in waveform, hyperactivated sperm showed little increase in beat frequency but a significant change in waveform. This increases the shear angle due to an increase in the amount of microtubule sliding, but not an increase of slide velocity (Ohmuro and Ishijima, 2006). These changes provide the sperm with an extremely exaggerated movement that helps them to penetrate the cumulus mass and zona pellucida (Parrish and First, 1993). The increase in the flagellar beat frequency may also contribute to the release of sperm bound to the oviductal epithelial cells (Suarez, 1998; Talevia and Gualtieri, 2001).

Nichol et al. (1997) showed that follicular and oviductal fluids have a significant influence on boar sperm motility. They found that there is an increase in the number of hyperactivated spermatozoa within the preovulatory ampullary-isthmus

junction fluid. This rise in sperm motility could be produced by the presence of certain substances produced during oestrus, and at the site of fertilization.

Although the events that regulate hyperactivation are still not completely understood, it is evident that hyperactivation is triggered by Ca^{+} at the flagellum axoneme level. In some species, it has been shown that this process involves an increase in intracellular cAMP. In humans, sperm hyperactivation may be produced by a rise in ampullar fluid Ca^{2+} concentrations, along with increases in intracellular cAMP (Wolf, 1996). Also, in monkeys, an initial increase in intracellular calcium, followed by a rise in cAMP, with a simultaneous Ca^{+} decrease (Ishijima et al., 2006), occurs during hyperactivation. A recent study demonstrated that flagellar hyperactivation in boar spermatozoa is produced by a calcium-sensitive PKC pathway in the connecting piece of the flagella. This process is regulated by cAMP-PKA signaling through the activation of both PLCγ 1 and PKC serine/threonine phosphorylation pathways (Harayama and Miyake, 2006). Contradictory to this study, Martínez and Suarez (2004) proposed that hyperactivation stimulated with caffeine and procaine in bovine sperm is initiated by Ca^{+} signaling but is not dependent on cAMP-PKA pathways. Collectively, one can assume that sperm hyperactivation is triggered by Ca^{+} signaling pathways and completed by cAMP signaling in several species.

Hyperactivation can be measured by computer-assisted semen analysis (CASA) or by manual evaluation (phase-contrast microscope). Since caffeine, procaine or glucose tend to stimulate hyperactive motility, these substances can be used to evaluate sperm hyperactivation *in vitro* (Parrish, et al., 1978; Brooks, 1990). Nevertheless, Ho and Suarez (2001) found that the hyperactivation induced by caffeine depends on external Ca^{2+} influxes, whereas hyperactivation by thapsigargin and thimerosal do not. It seems that these substances activated the use of Ca^{2+} stored in the sperm neck instead of external Ca^{2+} sources. Therefore, the presence of external Ca^{2+} in culture media has to be considered when hyperactivation is evaluated *in vitro*.

2.1.6. Sperm transport in the female tract

Spermatozoa are transported from their site of deposition in the female tract to the isthmus portion of the oviduct, through a combination of muscular contractions, female tract cilia movements, ejaculate or insemination fluid currents, and sperm motility (Hawk, 1983). Sperm are stored at this point, in what is referred to as the sperm reservoir, waiting for the release of the oocyte(s) from the ovary (Mburu et al., 1996, 1997).

During natural breeding, the deposition of the sperm occurs at different points in the female tract depending upon the species. Primates, ruminants, rabbits and hares deposit semen in the vaginal area, whereas dogs, horses, pigs, llamas and rodents ejaculate into the cervix and uterus (Scott, 2000). Similarly, the site of semen deposition for artificial insemination (AI) varies depending on the species being inseminated and the AI technique used. For example, in conventional swine AI (intra-cervical AI) deposits the sperm dose into the cervix, whereas with trans-cervical insemination techniques, the AI catheter traverses the cervix and places the sperm in the body of the uterus. Finally, for intrauterine or deep-uterine insemination techniques, sperm are deposited deep into one of the uterine horns (Hawk, 1993; Watson and Behan, 2002; Roca et al., 2006). The site of sperm deposition determines the number of barriers (cervix, uterus and utero-tubal junction) that the sperm must cross in order to enter the isthmus portion of the oviduct. This, in turn, affects the final number of spermatozoa reaching the site of fertilization (Scott, 2000; Watson and Behan, 2002). The number of spermatozoa required for successful fertilization also tends to be related to the sperm quality used (Sivashanmugam and Rajalakshmi, 1997; Amann et al., 2000; Thundathil et al., 2001).

Sperm transport in the female tract is considered to occur in two phases (Scott, 2000; Suarez and Pacey, 2006): 1) the first phase involves rapid transport that moves sperm within minutes of insemination into the oviduct. Rapid sperm transport is attributed mainly to muscular contractions, pressure changes in the lumen of the female tract and ejaculate or insemination fluid currents. This first sperm population

found in the oviduct is generally damaged and/or immotile; 2) The sustained phase of transport that follows involves a continuous migration of sperm to the oviduct. During this phase, competent sperm are transported through the utero-tubal junction (UTJ) to establish the sperm reservoir in the lower isthmus (Overstreet and Cooper, 1978). The UTJ is considered to work as a filter for abnormal sperm, but it seems that specific sperm surface proteins are also required for morphologically normal and motile sperm to pass through the junction (Suarez, 1987; Nakanishi et al., 2004; Suarez and Pacey, 2005).

2.1.7. Sperm reservoir

2.1.7.1. Sperm reservoir formation

There is a dynamic interaction between the sperm, luminal fluids and epithelial cells during their transit through the female tract that may help the healthy sperm to reach and colonize the lower isthmic region forming a sperm reservoir. The lower isthmic environment, where the sperm reservoir is established, seems to maintain sperm viability (Smith and Nothnick, 1997; Gualtieri and Talevi, 2000, 2003; Petrunkina et al., 2001b; Töpfer-Petersen et al., 2002), control sperm release to prevent polyspermy, and may control sperm capacitation and hyperactivation, thus synchronizing sperm fertilizing ability with the time of ovulation (Suarez, 1998; Scott, 2000; Petrunkina et al., 2001; Talevi and Gualtieri, 2001). The three major factors that influence sperm reservoir formation, as well as sperm release and transport to the fertilization site, are: A) sperm quality and the availability of sperm-specific membrane receptors (Rodríguez-Martínez et al., 1998; Petrunkina et al., 2001; Jelinkova et al., 2004; Rodríguez-Martínez et al., 2005; Liberda et al., 2006); B) oviductal epithelium cells that differ between oviductal sections (Rodríguez-Martínez et al., 1990; Yanz et al., 2006); and C) composition of the oviduct fluids, that varies between sections and changes throughout the estrous cycle (Johansson et al., 2000; Kumaresan et al., 2005, 2006; Yanz et al., 2006).

In several species it has been reported that the heads of sperm in the reservoir bind to the apical segment of the oviductal epithelium, resulting in an intimate sperm-epithelium interaction (Krishna, 1997; Suarez, 1987; Hunter et al., 1991). Furthermore, *in vitro* results have shown that only acrosome intact and uncapacitated spermatozoa are able to bind to the luminal epithelium and retain their viability for an extended periods of time until ovulation occurs (Gualtieri and Talevi, 2000; Green et al., 2001). Petrunkina et al. (2001) found that epididymal sperm showed lower oviductal binding compared to ejaculated sperm. This finding led them to the conclusion that seminal plasma components, acquired by sperm during ejaculation, play a role in sperm-oviduct interactions and are also important for maintaining sperm in an uncapacitated state.

Oviductal receptors, related to these sperm-oviduct cell interactions, appear to involve specific carbohydrate recognition sites that vary among species. Inhibitors of sperm binding have been found in different species, such as sialic acid and fetuin in hamsters (Suarez, 1998), asialofetuin and its terminal sugar galactose in stallions (Dobrinski et al., 1997; Suarez, 1998), fucose in bulls (Revah et al., 2000), in addition to maltose, lactose (Green et al., 2001) and mannose in pigs (Green et al., 2001; Wagner et al., 2002). These findings indicate that specific carbohydrates bind to the sperm membrane during ejaculation and play an important role in the sperm-oviductal binding. Confirming this hypothesis, recent studies (Jelinkova et al., 2004; Liberda et al., 2006) demonstrated that heparin-binding proteins from the spermadhesin family of seminal plasma proteins attach to the sperm plasma membrane during ejaculation and are involved in the binding of sperm to the oviductal epithelial cells. Based on these results, one can hypothesize that acrosome damaged and/or capacitated sperm have lost their sperm membrane proteins and will be unable to bind to the oviduct epithelial receptors and form a reservoir.

Several studies have been conducted in order to understand the role of the oviductal environment in sperm reservoir formation and sperm release. Research conducted in the human (Quintero et al., 2005) and bull (Boquest et al., 1998) suggests

that proteins secreted in the oviduct by the epithelial cells are influential in these processes. Oviductal apical plasma membrane preparations tested in pigs (Fazeli et al., 2003), horses (Dobrinski et al., 1997ab) and bulls (Gualtieri et al., 2005) were found to maintain sperm viability and motility, as well as protect sperm from a premature acrosome reaction. Smith and Nothnick (1997), working with the rabbit, showed that oviductal epithelial cells help to maintain sperm viability and suppress motility when the membranes of the oviductal epithelial cells and spermatozoa establish direct contact. Furthermore, a series of studies with equine sperm (Dobrinski et al., 1996, 1997ab) demonstrated that direct interactions between the sperm membrane and oviductal epithelial cells are needed to maintain the spermatozoa's low internal calcium levels (Ca^{2+}). In support of these results, Petrunkina et al. (2001) showed that pig sperm bound to the oviductal epithelial cells had a reduction in Ca^{2+} intake and reduced tyrosine phosphorylation of membrane proteins, which may prevent premature capacitation and acrosome reaction, thereby prolonging sperm viability.

Furthermore, proteins from different regions of the buffalo oviduct (isthmus and ampulla) and from cows at different points in their estrous cycle (luteal and non-luteal) have been added to the semen extenders used in freezing buffalo semen (Kumaresan et al., 2005). Post-thaw semen quality was dependent on the source of the oviductal proteins (region and cycle stage), with isthmus proteins from cows in the non-luteal phase having the most beneficial effects. It has been proposed that oviductal proteins reduce lipid peroxidation levels during or/and after cryopreservation (Kumaresan et al., 2006), protecting the sperm membrane from oxidative damage and resulting in better sperm quality after thawing. However, Fazeli et al. (2003), working with pig apical plasma membranes preparations and fresh sperm samples, did not find the same relationship. They found that maintenance of sperm viability in the oviduct was not influenced by the region (isthmus vs. ampulla) of the oviduct nor the stage of the estrous cycle (luteal and non-luteal phase).

Although further research in this area is necessary, all these studies demonstrate a role for the oviductal environment in maintaining sperm viability, inhibiting sperm motility, and delaying capacitation and the acrosome reaction. Collectively, this contributes to the maintenance of the sperm reservoir and ensures an adequate but not excessive number of competent sperm is available at the site of fertilization when needed.

The sperm's capacity to bind to the oviduct epithelium has been tested using sperm-binding assays *in vitro* and appears to be indicative of boar fertility, as well as changes in sperm quality during sperm storage (Waberski et al., 2006). It is important to note that the sperm populations found in the reservoir depends on sperm quality (ejaculate and/or boar), site of sperm deposition and number of sperm inseminated, all of which may reflect ejaculate and/or male fertility differences.

2.1.7.2. Sperm release

The sperm reservoir periodically releases small numbers of sperm that are transported by muscle contractions to the upper segments of the oviduct during the periovulatory period. Mburu et al. (1996) confirmed this in pigs by demonstrating that there is a re-distribution of sperm from the UTJ site to the upper portions of the isthmus before, during and after ovulation. The number of sperm present in the different segments of the UTJ, isthmus and ampulla changes gradually as ovulation occurs. These findings were in agreement with earlier studies with rabbits (Overstreet and Cooper, 1978) where sperm numbers increased in the upper segments of the isthmus at the time of ovulation. On the other hand, intermittent release of groups of sperm could be due to the presence of different bicarbonate-responsive sperm subpopulations (responsive and unresponsive to bicarbonate) (Satake et al., 2006). Each subpopulation could be activated at different points during the sperm reservoir maintenance. Also the bicarbonate-responsive subpopulation is affected by oviductal proteins and varies between boars, so this could be another factor to consider when evaluating male fertility *in vitro*.

At present, it is still uncertain whether specific secretions, sperm surface changes, and/or hyperactivation are responsible for sperm release. One hypothesis is that the sperm plasma membrane exhibits receptors that bind to the epithelial cells, and those are lost or modified during capacitation, allowing the sperm to be released from storage. Another proposal is that changes in concentrations of glycoconjugates during the oestrous cycle produce the increase in flagellar beat frequencies seen in oviductal bound-sperm, followed by release of a highly motile sperm from the oviductal epithelium (Talevia and Gualtieri, 2001). Nichol et al. (1997) showed that pre-ovulatory ampullary-isthmic junction fluid increases the number of hyperactivated spermatozoa. Therefore, hyperactivated motility may generate enough force to break the binding between sperm and oviductal epithelial cells as well as penetrate the cumulus cells and zona pellucida during fertilization (Suarez, 1998). However, recent studies using bull semen demonstrated that hyperactivation alone is not enough to release sperm adhering to the oviduct epithelium *in vitro*. They concluded that sperm plasma membrane remodeling during capacitation induced by heparin is mainly responsible for the release of sperm adhering to the oviduct epithelium (Gualtieri et al., 2005).

These studies suggest that changes in the oviduct environment (hormones, mucus, etc) related to ovulation may be responsible for the release and capacitation of the sperm maintained in the reservoir. Further evaluations need to be done to understand all the facts that play a role in sperm release and capacitation.

2.1.8. Acrosome reaction and fertilization

Before undergoing the acrosome reaction the sperm is involved in a series of events including penetration of the cumulus mass and, depending on the species, attachment and binding to the zona pellucida of the oocyte.

Three layers surround the mammalian oocyte: the cumulus mass, corona radiata and the zona pellucida. A hyaluronic-acid-rich matrix and the cumulus cells form the cumulus mass and corona radiata (Talbot, 1985). The initial step in the

sperm-oocyte interaction is sperm penetration through the cumulus mass and corona radiata. Hyaluronidase (PH-20), present in the sperm plasma membrane disperses the hyaluronic acid-rich matrix and the cumulus cells, allowing the sperm to reach the zona pellucida (Wolf, 1994; Li, 2002). Cherr et al. (1999) proposed that when the macaque sperm surface PH-20 receptor binds to the hyaluronic acid-rich matrix it produces an increase in intracellular Ca^{2+} that will prepare the sperm for the acrosome reaction that is induced by sperm-zona pellucida binding.

Sperm binding and the acrosome reaction are controlled by the species-specific receptors present in the zona pellucida, and the acrosome reaction occurs on or near the zona pellucida. The initiation of the acrosome reaction and sperm-zona pellucida binding may vary according to species. For example, in the guinea pig, acrosome reacted sperm can bind to the zona pellucida (Schroer et al., 2002), whereas in human and macaque sperm the acrosome reaction is induced after binding to the zona pellucida (review Yanagimachi, 1994; Cherr et al., 1999).

Studies using monoclonal antibody techniques have shown that the basic structure of the zona pellucida is formed by three glycoproteins: ZP1, ZP2 and ZP3 (ZPB, ZPA, and ZPC in pig, respectively (Dunbar et al., 2001). Each of these glycoproteins has a specific function. ZP2 is apparently a secondary sperm receptor (Mortillo and Wassarman, 1991). After fertilization, ZP2 changes its molecular composition (ZP2f) and, together with ZP3, provides a secondary block to polyspermy. The oligosaccharide (N-linked) component of ZP3 regulates the initial binding of the sperm to the zona pellucida, and its polypeptide (O-linked) component is needed to induce the sperm acrosome reaction (Yanagimachi, 1988; Wassarman, 1990; Wolf, 1994; Williams et al., 2006). The ZP1 macromolecules cross-link filaments of ZP2 and ZP3, forming the three-dimensional matrix of the zona pellucida (Wassarman, 1999a). Sperm-zona binding can be evaluated using the hemizona-binding assay (Wolf, 1994), the sperm zona pellucida binding assay (Fazeli et al., 1993), or *in vitro* maturation and fertilization techniques (Xu et al., 1998).

Several investigations have used lectins to demonstrate the presence of different zona-binding sperm receptors. For example, using these techniques, the binding of a 95KDa sperm membrane protein with ZP3, but not with ZP2, was confirmed. This binding stimulates the activity of a sperm tyrosine kinase, inducing the biochemical pathway of the acrosome reaction (Leyton and Saling, 1989).

After binding between sperm acrosome and the zona receptors, sperm undergo an acrosome reaction. The acrosome reaction is an exocytotic event that includes the fusion of the outer acrosomal membrane with the overlying plasma membrane to form vesicles, thus allowing for the escape of acrosomal enzymes.

The acrosome reaction process involves a series of events. Many of the acrosome reaction mechanisms have been studied, but the complete process is presently unclear. It is known that the interaction of the murine sperm plasma membrane receptor with ZP3 produces an increase of cAMP concentration in the sperm (Mortillo and Wassarman, 1991). Sodium-potassium ATPase is then inactivated by a high influx of external calcium which produces an efflux of H^+, increasing the intracellular pH. Calcium facilitates fusion of the plasma membrane and the acrosomal membrane, and the activation of the enzyme acrosin. Acrosin functions to break up the acrosomal matrix and causes several additional enzymes and lysins to be released (see reviews of Yanagimachi, 1988; Wassarman, 1990). Acrosin also acts as a secondary ligand molecule which helps to maintain the spermatozoa on the surface of the zona pellucida (Leggio et al., 1994). These enzymes and lysins, released by the action of acrosin, destroy the covalent and non-covalent bonds of the zona pellucida, helping the penetration of the sperm through this layer. The spermatozoa's internal acrosomal membrane remains and is attached at the equatorial segment (review Yanagimachi, 1988; Wassarman, 1990). In addition to the enzymes necessary for spermatozoa to cross through oocyte layers, sperm motility (hyperactivated motility) is also an important factor, providing the spermatozoa with the force necessary to cross these layers and reach the vitelline membrane (Wolf, 1994).

Several investigations *in vitro* have demonstrated that other molecules such as progesterone (Cheng et al., 1998), glycine, ionophore A23187, and arachidonic acid among others can induce the acrosome reaction (Zaneveld, 1996). Therefore, the ability for sperm to undergo the acrosome reaction can be evaluated *in vitro* and may be used to assess semen quality.

After passage of the spermatozoon through the zona pellucida and perivitelline space, it initially binds to the oocyte plasmalemma at the equatorial region and then at the postacrosomal region. The sperm and oocyte membranes then fuse, allowing the deposition of the sperm nuclear material into the oocyte cytoplasm. The spermatozoon nucleus integrated into the oocyte cytoplasm loses its membrane and starts decondensing (loss of the disulfide cross-links) and forms the male pronucleus, which migrates towards the female pronucleus. Finally, the fusion of the female and male pronuclei occurs (syngamy), forming the zygote that starts the embryonic mitotic phase (embryogenesis) (see review of Yanagimachi, 1988; Hing-Sing, 1994). Urner and Sakkas (1994) found that glucose is not only necessary for the terminal stages of capacitation and hyperactivated motility in the mouse, but also plays an important role in the fusion between sperm and oocyte membrane.

2.2. SEMINAL PLASMA

Seminal plasma is formed by secretions from the epididymis and the male accessory sex glands. The accessory sex glands include the ampulla, seminal vesicle, prostate, bulbourethral (or Cowper's) glands, along with the urethral glands (or Littré's gland), but not all these glands are present in all mammals. For example, the ampulla is not present in the boar and the seminal vesicles are absent in dogs and cats (Mann, 1964, as cited by Shivaji et al., 1990).

There are several physiological functions of seminal plasma. First of all, it transports spermatozoa from the male tract into the female genital tract. Spermatozoa, as discussed, are produced in the testes then matured and stored in the epididymis. During ejaculation, the sperm are mixed with epididymal secretions, transported from the cauda epididymis through the deferent ducts to the urethra, where they are mixed with the secretions of the accessory sex glands to finally be ejected inside of the female tract by muscular contractions of the urethra. Seminal plasma also acts as a buffer solution. It is well known that the slightly basic pH of the seminal plasma is useful to neutralize the acid pH of the female tract (Hing-Sing, 1994). Mann and Lutwak-Mann (1981) demonstrated that ions such as Na^+, K^+, Ca^{2+}, Mg^{2+}, etc, produce 50% of the buffering capacity of human semen, whereas proteins and HCO^-_3/CO_2 systems produce just 25% (Mann and Lutwak-Mann, 1976; Wolters-Everhardt et al., 1986, 1987). Seminal plasma is also a source of nutrients, providing the energy substrates (glucose, fructose, etc) that increase the spermatozoa's life span (Shivaji et al., 1990). Binding of specific seminal plasma proteins to the sperm plasma membrane is involved in sperm-oviductal binding, prevention of early capacitation, maintenance of sperm viability, sperm-zona pellucida interactions and oocyte-sperm binding (Shivaji et al., 1990; Veselsky et al., 1999; Töpfer-Petersen et al., 1998; Jonáková et al., 2000; Petrunkina et al., 2001; Maňásková et al., 2003; Liberda et al., 2006). At the uterine level, specific seminal plasma proteins seem to control the neutrophil response, as shown in the mouse (Tremellen et al., 1998), pig (Rozeboom et al., 1998, 1999, 2001) and rat (Assreuy et al., 2002, 2003). Furthermore, there is evidence that some seminal plasma components play an active role in the female reproductive tract of the pig, such as enhancing uterine contractions (Claus et al., 1990; Troedsson et al., 2005), advancing ovulation (Claus, 1990; Waberski et al., 1995, 1997, 2000, 2006), and elimination of non-viable spermatozoa (Troedsson et al., 2005).

2.2.1. Composition of seminal plasma

Seminal plasma composition and volume differ between species, individuals (Mann and Lutwak-Mann, 1981, as cited by Shivaji et al., 1990), ejaculates from the

same individual, and even between different fractions of individual ejaculates in the boar (Zhu et al., 2000; Zhu, 2000ab). In general, seminal plasma is composed of organic and inorganic chemical components as listed in Table 2-2. However, proteins are the major components of seminal plasma and their role during the fertilization process will be reviewed.

2.2.2. Seminal Plasma Proteins

Proteins in seminal plasma play an important role in different events associated with fertilization, such as sperm motility, capacitation, acrosome reaction, formation of the sperm reservoir and sperm-oocyte interactions (Shivaji et al., 1990; Liberda et al., 2006; Pilch and Mann, 2006). In order to facilitate the study of these proteins they have been classified as follows: 1) spermadhesins, 2) sperm motility inhibitory proteins and 3) decapacitation factors.

2.2.2.1. Spermadhesins

The predominant proteins in seminal plasma are from the spermadhesin family, representing 75% of the total protein of the seminal plasma. Spermadhesins are secretory proteins with a molecular weight ranging from 12 to 20 kDa, built in a single CUB domain architecture with an amino acid sequence homology of about 40 to 60% (Ekhlasi-Hundrieser et al., 2002; Töpfer-Petersen et al., 1998). Spermadhesins have been identified in the stallion, bull and boar, as presented in Table 2.3a-b. The spermadhesins identified in boars are AWN, AQN-1, AQN-3, PSP-I, and PSP-II, and glycosylated isoforms of AWN, AQN-3, PSPI, and PSP-II (monomers and dimmers). These proteins coat the sperm plasma membrane during their transit through the epididymis and during ejaculation, producing structural changes to the sperm surface. In general, they are considered to maintain plasma membrane stability, play important roles in the sperm-oviduct interactions (Maňásková et al., 2003; Petrunkina et al., 2001b; Liberda et al., 2006), capacitation, spermatozoa-zona binding (Veselsky et al., 1999; Töpfer-Petersen et al., 1998) and sperm-oocyte Töpfer-Petersen et al., 1998; Jonáková et al., 2000). Metz et al. (1990) demonstrated that sperm from vesiculectomized boars adsorb large quantities of seminal plasma proteins, and

particularly proteins with low molecular weight. Spermadhesins, in boar seminal plasma, are mainly produced by the seminal vesicles but some are also produced by the epididydimis, prostate and testes (Ekhlasi-Hundrieser et al., 2002). The Spermadhesins AQN-1, AQN-3, AWN and free PSP-I exhibit the ability to bind to heparin molecules, while PSP-II, PSP-I/PSP-II heterodimers and some PSP-I do not have that characteristic (Calvete et a., 1996a; Jonáková et al., 1998; Solis et al., 1998; Maňásková et al., 1999). Spermadhesins tend to associate and form complex molecules that could be responsible for their varying biological functions along the fertilization process.

2.2.2.1.1. AWN

AWN is a spermadhesin glycoprotein with 133 amino acid residues and a molecular weight from 14 to 17 kDa (Sánz et al., 1992; Veselsky et al., 1999). AWN is named after its first amino acid residues (Ala-Trp-Asn) and is synthesized by the rete testis, prostate, seminal vesicles (Sinowatz et al., 1995) and female reproductive tract (Ekhlasi-Hundrieser et al., 2002). Two isoforms of AWN have been described, AWN-1 and AWN-2. Both AWN isoforms were found attached primarily to the sperm membrane, overlying the acrosomal region and peripherally to the plasma membrane as detected by membrane carbohydrate-recognition (Dostálová et al., 1995; Calvete et al., 1996b). They also bind to β-galactosidases (Calvete et al., 1996; Ekhlasi-Hundrieser et al., 2005) and to a O-linked oligosaccharides of the zona pellucida (Dostálová et al., 1995; Calvete et al., 1996b). AWN-1 is the only spermadhesin present on the epididymal spermatozoa (Dostálová et al., 1995), which suggests a possible zona-pellucida-binding activity (Sánz et al., 1992; Rodríguez-Martínez et al., 1998). Confirming this Rodríguez-Martínez et al. (1998) found that AWN remains attached to the sperm until they bind to the zona pellucida. The presence of AWN in the remnants of plasma membranes of sperm bound to the zona pellucida were detected in collected embryos. Also, AWN may also have a role in the capacitation process as suggested by its heparin binding capability (Töpfer-Petersen et al., 1998; Calvete et al., 1996a, 1997). Furthermore, AWN does not bind to mannose species and when added to oviduct explants it does not inhibit sperm binding to the

epithelial cells (Ekhlasi-Hundrieser et al., 2005). The low levels of AWN attached to the surface of live ejaculated spermatozoa (Petrunkina et al., 2000) suggest that it is not primarily responsible for the sperm-oviductal interaction during the sperm reservoir formation.

2.2.2.1.2. AQN

The AQN protein of the spermadhesin family, like AWN, was named after its first amino acid residues (Ala-Gln-Asn) (Veselsky et al., 1999). Three AQN subunits have been identified, AQN-1, AQN-2 and AQN-3. Recent studies, confirmed that AQN-1 and AQN-3 bind to α- and β-galactose and α-mannose structures, that have been related with the oviductal epithelium and zona pellucida binding, respectively (Calvete et al., 1996b; Ekhlasi-Hundrieser et al., 2005). Ekhlasi-Hundrieser et al. (2005) found that during the first 30 min of capacitation there is a reduction in AQN-1 and the mannose binding sites in the viable sperm. During this time there is a decrease in the sperm-oviduct binding, while the sperm galactose-binding sites increase more than 50% as well. They also confirmed that exposure of AQN-1 in oviductal explants inhibit sperm-oviductal binding whereas AWN does not. Liberda et al., (2006) demonstrated that mainly AQN-1 attached to the oviductal epithelium cells in the ampulla, isthmus and uterine tubal junction regions via mannose-mediated interaction and this interaction was affected by oviductal fluid. Those results support the hypothesis that AQN-1 plays an important role in sperm-oviductal interactions, sperm reservoir formation, mediated by carbohydrate recognition in a similar way as its bovine counterpart, the seminal plasma protein PDC-109.

2.2.2.1.3. Porcine Seminal Plasma proteins

Porcine Seminal Plasma protein (PSP) was identified by Rutherfurd et al. (1992) as two subunits, PSP-I and PSP-II, with 80% homology. Both, PSP-I and PSP-II are N-glycosylated proteins composed of 110 and 116 amino acids (Nimtz et al., 1999; Rutherfurd, 1992) with a 12 to 14 kDa molecular weight, respectively (Rutherfurd, 1992; Zhu, 2000b). PSP-I may prevent premature capacitation and acrosome reaction (Kwok et al., 1993; Töpfer-Petersen et al., 1998), and have

immune-regulatory activity in the uterine environment (Yang et a., 1998; Assreuy et al., 2002; Assreuy et al., 2003). Multiple forms of PSP-I involve differences in carbohydrate moieties, monodimer and heterodimer associations that could be responsible for its variety of functions (Rutherfurd., 1992; Nimtz et al., 1999). PSP-II has mainly been recovered from the non-heparin binding seminal plasma fraction and is considered to be the primary component of the non-heparin binding fraction (Calvete et al., 1995). PSP-II exhibits the capacity to bind to the glycoproteins of the zona pellucida and also shows affinity for soybean trypsin inhibitors, and so could be involved in initial spermatozoa-oocyte recognition and the acrosome reaction (Calvete et al., 1995; Solis et al., 1998). Furthermore, PSP-II has been associated with PSP-I forming a non-covalent heterodimer that is found in the non-heparin fraction of SP, although both subunits are capable of binding heparin. PSP-I/PSP-II heterodimers bind weakly to zona pellucida glycoproteins (Calvete et al., 1995), and are involved in a pro-inflammatory response, stimulating macrophages to secrete a neutrophil chemotactic substance to initiate an immune response (Assreuy et al., 2002; Assreuy et al., 2003). Recent studies have demonstrated that exposure to PSP-I/PSP-II heterodimer maintains sperm motility, viability, and mitochondrial activity in highly extended ejaculates (Centurion et al., 2003; García et al., 2003; Caballero et al., 2006) as well as in frozen semen (Caballero et al., 2004). Furthermore, zinc ions (Zn^{2+}) and an acidic pH produce low rates of PSP-I/PSP-II heterodimer dissociation (Campanero-Rhodes et al., 2005). Exposure of the dissociated PSP-I to Zn^{2+} produces unfolding and reorganization of the PSP-I molecule (Campanero-Rhodes et al., 2006), thereby recovering its heparin-binding capabilities which are masked when it is in a heterodimer association. Conversely, PSP-II is not affected by the action of this cation. Such effects could be relevant to the PSP-I protein's activities at the uterine level.

2.2.2.2. Decapacitation factors

Decapacitation factors present in the seminal plasma are generally proteins of high molecular weight that coat the sperm plasma membrane during ejaculation to prevent premature capacitation (Oliphant et al., 1985 as cited by Shivaji et al., 1990;

Bonilla et al., 1996). The removal of decapacitation factors produce changes in the plasma membrane that altered the function of sperm receptors, providing the starting point for the capacitation processes (Table 2-3b). Bonilla et al. (1996) reported a protein, with a molecular weight greater than 100 kDa produced in pig seminal plasma, which inhibited the acrosome reaction. In humans, three glycoproteins of approximately 5, 70, and 200 kDa are associated with decapacitation factors. Lee and Wei (1994) also identified a series of protease inhibitors that act on specific proteases known to be activated during capacitation and the acrosome reaction. Scott (2006) found that one of the coating factors could be a galactose and N-acetylglucosamine polymer that binds to the sperm membrane receptor glycosyltransferase. Thus, decapacitation factors present in seminal plasma bind to sperm surface during ejaculation, providing temporary protection from premature capacitation and acrosome reaction while they traverse the female tract.

2.2.2.3. Sperm motility inhibitory proteins

Sperm motility inhibitors have been identified in bull, rat, rabbit, human and boar seminal plasma (de Lamirade and Gagnon, 1984). The human seminal plasma motility inhibitor (SPMI) is a semenogelin produced by the seminal vesicles in a precursor form with a molecular weight of 52kDa. SPMI precursor inactivates sperm motility in a concentration dependent manner as demonstrated by Robert and Gagnon (1996) and is degraded after ejaculation by prostatic proteases (Yoshida et al., 2003). Another motility inhibitor factor was first identified in boar seminal plasma by Iwamoto et al. (1992) as a 50 kDa protein. Later, using gene cloning techniques (Iwamoto et al., 1995) its composition was determined as an aggregation of three polypeptides (12 to 18 kDa) and these polypeptides exhibited an identical composition to the AQN-3 spermadhesin, except for two cDNA positions (Iwamoto et al., 1995; Yamagimachi, 1998). Therefore, the binding of associated AQN-3 proteins to the sperm membrane produces sperm motility inhibition that is reestablished when these molecules are removed from the sperm surface during capacitation.

2.2.3. Seminal plasma proteins and male fertility

As presented above, seminal plasma proteins play an important role in the fertilization process. Therefore, several studies have investigated the action of these proteins before and during fertilization. Currently, with the use of proteomics, specific proteins in seminal plasma have been identified as potential markers of male fertility in several species. Killian et al. (1993) identified two seminal plasma proteins correlated with high fertility in bulls (26 kDa, pI 6.2 and 55 kDa, pI 4.5) and two proteins that were correlated with low fertility (16 kDa pI 4.1 and 16 kDa pI 6.7). The 55 kDa fertility-associated protein has since been identified as osteopontin (Cancel, 1999) and the 26kDa fertility-associated protein as lipocalin-type prostaglandin D synthase (Gerena et al., 1998). In a study with stallions, Brandon et al. (1999) reported that SP-1, a 72 kDa, pI 5.6 seminal plasma protein, was positively correlated with fertility and demonstrated its antigenic homology with the bull 55 kDa fertility-associated protein, osteopontin. Furthermore, three other proteins (75 kDa, pI 6.0; 18 kDa, pI 4.3; 16 kDa, pI 6.5) were found to be negatively correlated with stallion fertility. In the boar, Flowers (2001) identified two boar seminal plasma proteins (55 kDa, pI 4.5 and 26 kDa, pI 6.2) that were positively associated with *in vitro* and *in vivo* fertility. Earlier, Flowers (1997) demonstrated that mixing seminal plasma of high fertility ("dominant") boars with the sperm of low fertility ("non-dominant") boars improves the fertility of the sperm used for *in vivo* fertilization. Furthermore, a significant reduction in fertility is observed when sperm of dominant boars was exposed to seminal plasma of non-dominant boars. Likewise, Zhu et al. (2000) found differences in oocyte penetration rates *in vitro* when spermatozoa were pre-incubated with different fractions of seminal plasma from the same ejaculate. In the same study, assessing the qualitative and quantitative characteristics of sperm binding proteins in the seminal plasma, Zhu et al. (unpublished data) found a negative correlation between the amount of seminal plasma proteins and fertility. These studies confirm the direct effect of seminal plasma components on sperm quality, as determined by differences in fertility *in vivo* and *in vitro*.

Several studies have determined that the functional state of sperm is reduced during the freezing-thaw process and during the cooling storage of fresh semen (Pérez-Pé et al., 2002; Petrunkina et al., 2005b) as well as during the sex sorting process (sperm highly diluted) (Maxwell et al., 1998, 1999). Cold-shock induces changes in the plasma membrane and induces protein tyrosine phosphorylation that can accelerate the capacitation process and reduces spermatozoa fertilizing capacity and lifespan. The positive effect of including seminal plasma during these processes has been demonstrated, increasing sperm viability and reducing protein phosphorylation (Pérez-Pé et al., 2002; Petrunkina et al., 2005b).

Collectively, this information demonstrates the important role of seminal plasma, and particularly seminal proteins, during the fertilization process. For this reason, it would be beneficial to identify fertility markers present in seminal plasma and develop a practical analysis that could become a part of the assessment of semen quality and prediction of male fertility.

2.3. TECHNIQUES FOR SEMEN EVALUATION

2.3.1. Semen evaluation and fertility

Currently, in the animal production industry, the routine semen evaluation commonly used during semen processing in AI centers (color, odor, concentration, morphology, viability, and motility) only detects male reproductive disorders that result in low fertility. However, these parameters are not useful for predicting relative fertility in healthy boars with ejaculate quality that meets normal industry standards (>70 % motility and <30% abnormal sperm morphology) (Flowers, 1995; Berger and Parker, 1989; Flowers, 1997; Flowers and Tuner, 1997; Xu et al., 1998; Popwell and Flowers, 2004), even though the productivity of these boars may still be substantially different and becomes particularly apparent when low sperm doses are used (Watson and Behan, 2000; Tardif et al., 1999; Mezalira et al., 2006). For this reason, in the last decade, several new technologies have been used to evaluate ejaculate (sperm and

seminal plasma) characteristics in order to find a comprehensive semen evaluation process that helps to predict semen quality and boar fertility potential.

2.3.2. Assessment of semen characteristics

Semen has several characteristics that can be evaluated by macroscopic, microscopic as well as functional analyses. All of these analytical approaches are outlined in the following sections.

2.3.2.1. Semen macroscopic analysis

2.3.2.1.1. Colour

Semen is normally opaque, chalky, and creamy in colour. A yellow, pink, brown or red coloration is abnormal. A yellow colour is generally the result of urine contamination; a pink, brown or red colour is often produced when red blood cells are present in the ejaculate. Visual observation is an adequate method of measuring this parameter (Diehl, 1984; Kubus, 1993; Brooks, 1990; Mortimer, 1994).

2.3.2.1.2. Odour

Some people do not detect any odor in normal semen, while others find that normal semen has a strong characteristic odour. Abnormal odours, such as those resulting from urine or putrefaction (preputial sac content), should be noted. The method of measuring this parameter is by olfactory detection (Kubus, 1993; Mortimer, 1994; Almond et al., 1994).

2.3.2.1.3. Volume

This parameter varies among species (Table 2-4). The volume should be assessed by weight (1g = approximately 1mL) or using a standard graduated volumetric device. Low volumes can be associated with disturbed secretory function of the accessory sex glands or a high frequency of ejaculation (Brooks, 1990; Kubus, 1993; Mortimer, 1994; Almond et al., 1994).

2.3.2.1.4. pH

The pH also varies among species (Table 2-4), but the normal semen pH ranges from 7.2 to 7.8. Semen pH is measured with a pH meter or pH paper strips. The prostatic secretions are acidic and seminal vesicle secretions are alkaline, so alterations in this characteristic may be indicative of accessory sex gland dysfunction (Parrish et al., 1978 and references therein; Brooks, 1990). The slightly basic pH of the seminal plasma has a buffering capacity that neutralizes the acidic pH of the female genital tract, protecting spermatozoa from a drastic change in pH (Hing-Sing, 1994). For bulls and dogs, the low pH of the caudal epididymal fluid could be responsible for sperm motility inhibition at this level (Carr et al., 1985).

2.3.2.1.5. Temperature

Ejaculate temperature ranges from 38 to 37 °C. Sperm are sensitive to drastic changes in temperature; therefore, ejaculate temperature has to be maintained from collection and all the way through the evaluation process and extension. After semen is protected with an extender solution the storage temperature most be reached gradually. During semen processing (dilution and storage) and AI, temperature fluctuations can damage the spermatozoa membrane integrity (Bamba and Cran, 1985, 1988; Moran et al., 1992). Therefore, drastic changes in temperature must be avoided at any time from collection until insemination.

2.3.2.2. Semen microscopic analysis

The microscopic analysis of the ejaculate is done using different kinds of microscopy equipment. As mentioned before, at a commercial level, the most common microscopic characteristics evaluated are: concentration, morphology, viability, and motility. Currently, for research purposes, more sophisticated techniques such as *in vitro* maturation and fertilization (IVM-IVF), fluorescence technologies (Juonala et al., 1999); *in vitro* cell (Dobrinski et al., 1997ab; Smith and Nothnick, 1997; Fazeli et al., 2003; Gualtieri et al., 2000, 2005) tissue culture (Petrunkina et al., 2001; Waberski et al., 2005, 2006); flow-cytometric methods

(Hansen et al., 2002) and others have been used for assessing sperm quality and potential male fertility.

2.3.2.2.1. Sperm concentration

Sperm concentration is a quantitatively indirect evaluation of spermatogenesis (Mortimer, 1994) that varies among species, breeds and individuals (Table 2-4). Currently, various methods are being applied to determine sperm concentration. These include the use of: 1) manual counting chambers, such as hemocytometers, which are generally considered to be the "Gold Standard", as well as Makler chamber, Microcell chamber or Bürker chamber, that have similar principles to the hemocytometer; 2) electronic instruments like spectrophotometers or colourimeters, that measure the amount of light absorbed by each semen sample (more concentrated samples absorb more light and vice versa); 3) currently, more sophisticated and expensive instruments like computer-assisted sperm analysis (CASA), Sperm Quality Analyzer (SQA) IIC (Kubus, 1993; Almond et al., 1994) or flow-cytometric microscopes (Hansen et al., 2002) have been tested for their accuracy in measuring sperm concentration, which due to their elevated costs are mainly use for research purposes or human fertility laboratories.

2.3.2.2.2. Sperm motility

Sperm motility and hyperactivation are important characteristics used to assess spermatozoa's potential to move through the female genital tract and penetrate the oocyte layers. Sperm motility is an indirect measurement of sperm viability and can be measured by subjective observation of sperm viewed under a microscope or by more objective measurements using CASA, Markler Counting Chamber, videotape recording, or photographic systems.

Sperm motility may be scored visually to determine the percentage of sperm presenting progressive motility and the overall percentage of motile sperm. There are several scoring systems to assess sperm motility; one of them is the 0 to 4 scale of progression rating. In this system the characteristics of sperm progression are

measured on a scale from 0 to 4: 0 means no motile sperm or dead sperm; 1 being no progressive sperm motility; 2, slow-to-fair progression; 3, good progression; and 4, very vigorous forward progression of motile sperm (Mortimer, 1994; Almond et al, 1994). Another method to score progressive and/or total sperm motility is by using a microscope with a final magnification of 200 x, screening at least five different fields and subjectively scoring to the nearest 5%. Visual sperm motility assessment requires training and practice in order to reduce human error during the evaluation.

Automated sperm motility evaluation by CASA evaluates general motility and forward motility as well as a series of specific sperm movements such as the curvilinear velocity (VCL), the straight line velocity (VSL), and amplitude of lateral sperm head displacement (ALH). The objective and accurate measurement of specific sperm movements could give more predictive information of sperm functional ability. For example, ALH is an important characteristic since the degree of lateral sperm head displacement regulates the flagellar wave amplitude, which gives the sperm the force to penetrate the oocyte barriers. Therefore, sperm with low ALH may have reduced fertility *in vivo* (Aitken, 1989).

Overstreet et al. (1992) suggested that CASA is the most objective and precise method to make measurements of sperm motility and specific sperm movements. Also with this tool, it is possible to measure sperm concentration (Brooks, 1990) and sperm morphology (normal range of sperm head size and shape) within major limits. However, as a new system, CASA has several factors that need to be considered in order to improve its accuracy, sensitivity and repeatability in order for it to be used as a reliable technique to predict semen quality and male fertility. This system has some limitations, such as the misclassification of debris as sperm cells in semen samples with low concentration. On the other hand, semen samples with high concentrations of sperm may have collisions that produce overlap of sperm trajectories that will be misinterpreted by the computer. Due to the size of sperm, tail abnormalities are not easily assessed by the CASA system. Therefore, a high level of training and/or experience is required to operate this equipment in order to detect and correct any

misclassification done by the computer program. The high cost of CASA may limit its use as a common tool to assess semen quality in commercial AI centers. There is another automatic sperm analyzer that has been used in human sperm evaluations called Sperm Quality Analyzer (SQA) IIC, which is a low-cost instrument, in comparison with CASA, that uses electro-optics, computer algorithms and video microscopy to perform semen evaluation rapidly and efficiently (less than 75 seconds per sample): This system also provides repeatable results, regardless of semen preparation (fresh, frozen, and treated semen) or operator. Suzuki et al. (2002), comparing the analyser system SQA with CASA found significant correlations of sperm motility, sperm concentration, sperm motility index parameters, between the two systems. However, further investigations are needed to consider the SQA IIC as a reliable test for animal semen evaluation.

Normal sperm motility is important for passage across barriers such as the female genital tract, cumulus mass and zona pellucida. There are many factors that affect sperm motility, such as temperature (Comhaire and Vermeulen, 1996), pH changes, and diverse substances. It is known that changes in environmental pH can either suppress or stimulate sperm motility (Gatti et al., 1993, Holm and Wishart, 1998). White (1993) observed that media with high levels of calcium and low temperature produce a decrease in sperm motility. Caffeine and glucose both stimulate sperm motility and have been used to evaluate this parameter and the potential of sperm to undergo hyperactivation *in vitro* (Parrish et al., 1978). Therefore, during sperm motility evaluation a series of important factors must be considered in order to reduce the chance of under or over estimations for a sample: 1) Temperature of the semen sample at the time of evaluation (36-37°C), pre-warm all the equipment used for the evaluation; 2) size of the sample must be standardized according to the size of the cover slide used (9 to 15 μL drop); 3) sperm concentration between samples; and 4) the use of caffeine.

Flowers (1997) concluded that the percentage of motile sperm gives a qualitative value to assess semen fertility and is only useful to estimate the

reproductive performance of boar semen with less of 60 % motile spermatozoa (Table 2-5). Xu et al. (1998) found no significant correlation between sperm motility and litter size, using 3 and 2 billion sperm per AI dose in pigs. However, Tardif et al. (1999) found that using 0.3 x 10^9 sperm per AI dose, the percent of sperm with normal motility was positively correlated with farrowing rate (r=0.783, P=0.01). This suggests that the use of large numbers of sperm per AI dose might mask actual boar fertility potential.

In contrast to motility estimates in raw or extended semen on the day of collection, sperm motility in extended semen at Day 7 has been correlated with *in vitro* fertility estimates (Xu et al., 1996b). However, its correlation with *in vivo* fertility has been variable (Juonala et al., 1998; Xu et al., 1998; Sutkeviciene et al., 2005). Therefore, the motility of extended semen at different days of storage may offer a practical and inexpensive approach to identifying relative boar fertility. However, more studies are needed to confirm this relationship and should involve precise methods of measuring sperm motility and motility characteristics, like CASA or SQAIIC.

2.3.2.2.3. Spermatozoa viability

A viable sperm is considered to be one that is alive and capable of fertilization. In order to estimate sperm viability, vital stains and the hypoosmotic-swelling test (HOST) are used. In general, vital stains estimate the sperm membrane's capacity to avoid the entrance of dyes, while HOST evaluate the sperm's capacity to resist swelling under hypo-osmotic conditions (Conmhaire and Vermeulen, 1996).

Vital Live-Dead stain can be useful to differentiate between dead spermatozoa and non-motile spermatozoa. Eosin-nigrosin, Trypan blue or Giemsa are some of the stains used to assess semen viability. These methods are based on the principle that damage to the membrane and/or the absence of an osmoregulatory system in dead spermatozoa allows penetration of the dye, while live sperm cells repel the stain. For this reason stained sperm are considered dead and the non-stained sperm are

considered to be alive (Brooks and Webster, 1990; Conmhaire and Vermeulen, 1996). Fluorescent vital stains are also used to evaluate the percentage of dead sperm. Some of these stains are: Propidium iodide (PI) in combination with carboxyfluorescein diacetate (CFDA) or SYRB-14 (SYBR). Propidium iodide is a DNA-specific stain (red) that does not penetrate the plasma membrane of intact cells, following similar principles as the vital stains. The other two fluorescent stains are membrane-penetrating stains that are used as membrane integrity markers. When spermatozoa exhibit partial or complete red nuclear staining (PI positive) they are considered to be dead, while spermatozoa that exhibit a complete green stain (CFDA or SYBR positive) are considered to be alive (Brito et al., 2003).

The hypo-osmotic-swelling test (HOST) assesses the osmoregulatory ability of the sperm membrane under hypo-osmotic environment (i.e. the functional integrity of the sperm membranes) environment (Comhaire and Vermeulen, 1996; Vázquez et al., 1997; Gadea and Matas, 2000; Brito et al., 2003). Dead sperm with damage to their plasma membrane lose the ability to control swelling. On the other hand, dead sperm with plasma membrane integrity and senescent spermatozoa (sperm becoming old) have a reduction in their osmoregulatory ability that results in uncontrolled swelling, which produces a rupture of the plasma membrane and loss of their swollen appearance during the test (Mortimer, 1994). Comparisons have been made between vital stains and HOST for the evaluation of sperm membrane integrity, and they have found that the HOST results had little or no correlation with the vital stain results (Eosin-nigrosin, Trypan blue, IP-CFDA and PI-SYBR). These differences are likely due to the fact that sperm characteristics measured by HOST are quite different from the vital stains. HOST evaluates the sperm membrane's ability to regulate the flux of electrolytes and non-electrolytes, and sperm plasma membrane integrity, while vital stains simply evaluate membrane integrity and not its biochemical activity (Vázquez et al., 1997; Brito et al., 2003).

Several studies, have demonstrated that sperm viability results, obtained with vital stains or fluorescent vital stains, have no significant correlation with fertility *in*

vitro in swine (Berger et al., 1996) and cattle (Brito et al., 2003), or *in vivo* in the pig (Juonala et al., 1999). Nevertheless, there is evidence demonstrating positive correlations between the HOST and zona pellucida penetration rate in human studies (Jeyendra et al., 1984), as well as *in vitro* fertilization parameters in pigs (Gadea and Matas, 2000) and cattle (Brito et al., 2003). Unfortunately, HOST results have little or no correlation with *in vivo* fertility in bulls (Correa et al., 1997) and boars (Pérez-Llano et al., 1998; Pérez-Llano et al., 2001).

In summary, HOST may be useful method to assess fertility *in vitro*, but are not a reliable test to predict male fertility *in vivo*.

2.3.2.2.4. Spermatozoa morphology

Sperm morphology is evaluated using different techniques and stains, such as Eosin-nigrosin, Trypan Blue, Giemsa, Papanicolaou, and Diff-Quik (Kruger et al., 1996). Since some of these stains can be imprecise, an alternative method is to fix the sperm sample with formalin or formal citrate and analyse the sample using a phase contrast microscope. Other techniques used for assessing sperm morphology include the use of transmission electron microscopy or CASA.

Sperm morphological analysis is an indirect evaluation of spermatogenesis and the sperm maturation processes. Sperm abnormalities can be categorized based on their origin such as: 1) primary abnormalities that are generally considered to be of testicular origin (spermatogenesis) (e.g. head and tail abnormalities) and 2) secondary abnormalities that are thought to be of epididymal origin (epididymal maturation defects such as the presence of cytoplasmic droplets) (Kubus, 1993; Briz, et al., 1995; Senger, 1999). For evaluation purposes, the morphological forms are often categorized based on the sperm anatomical region (head, midpiece and tail) as illustrated in Figure 2-8.

Berger and Parker (1989) did not find any correlation between spermatozoa morphology and boar fertility in a competitive mating situation in vivo. However, Xu

et al. (1998) found that morphological characteristics are a useful tool for assessing semen quality in boars. In this latter study, normal sperm morphology was considered to be responsible for part of the variance in litter size born when 2 billion normal and motile sperm per AI dose was used.

Another important morphological sperm characteristic is the integrity of the acrosome. This characteristic is assessed by acrosome stains such as Hoechst 33348, Hoechst 33258, SYBR-14, indirect immunofluorescence assay, chlortetracycline, and the *Pisum sativum* agglutin fluorescein assay. A practical alternative method to evaluate acrosome integrity is to use formal citrate solution and evaluating the fixed semen sample under a phase contrast microscope (Figure 2.8) to obtain the percentage of spermatozoa with acrosomal integrity.

2.3.2.2.5. Sperm DNA evaluation

In recent years, changes in sperm DNA structure have been evaluated as a cause of male infertility. Damage to spermatozoa DNA may occur at different points in the male reproductive tract (testicle or epididymis) as well as during semen collection, semen processing (semen extension for freezing) and semen storage, and could have a negative impact on fertilization and fetal development (Everson et al., 1994; Pérez-Llano et al., 2006; Fraiser and Strzezek, 2005; Boe-Hansen et al., 2005). Techniques used to evaluate DNA and chromatin quality are Comet, TUNEL, sperm chromatin structure assay (SCSA) and the sperm chromatin dispersion test. The main objective of all these tests is to evaluate the degree of DNA fragmentation in semen samples in order to assess semen quality. Strong positive correlations have been found between DNA fragmentation index (DFI) assessed by SCSA in boar semen and routine semen evaluation parameters such as motility (%), normal acrosome (%), abnormal sperm (%) and cells positive to HOST (Pérez-Llano et al., 2006). Sperm DNA damage increases during storage of raw and extended semen (Boe-Hansen et al., 2005; Pérez-Llano et al., 2006), as well as during sperm freezing-thawing processes (Fraiser and Strzezek, 2005). Sperm DNA fragmentation could be produced by the action of the endogenous reactive oxygen species (ROS) over the sperm membrane.

The release of acrosomal enzymes and the presence of active nucleases from the apoptotic process could also be directly or indirectly responsible for the DNA damage (Pérez-Llano et al., 2006). Differences in sperm DFI have been found between different boar breeds (Boe-Hansenet al., 2005) and between individuals of the same breed (Pérez-Llano et al., 2006); therefore, DNA evaluation could be used as an additional test for assessing semen quality and boar fertility.

2.3.2.3. Tests used to evaluate sperm function

Several investigators have developed tests that are useful for evaluating sperm functions that are directly related to the fertilization process, such as the ability to undergo capacitation, hyperactivation, acrosome reaction, membrane fusion, sperm zona pellucida penetration and/or oocyte fertilization. These tests are the sperm-zona pellucida binding test, sperm penetration assay (zona-free hamster egg, and homologous *in vitro* penetration assay), *in vitro* fertilization assay and sperm binding assay.

2.3.2.3.1. Sperm-zona pellucida binding test

The ability of sperm to bind to the zona pellucida can be evaluated using the hemizona assay (HZA) or the sperm zona pellucida binding assay. These assays measure the sperm's ability to approach and attach to the homologous zona pellucida of immature eggs (species-specific). In the hemizona assay, the zona pellucida is separated from the eggs and cut in two halves. Semen samples to be tested are incubated under capacitating conditions. One half of the zona is incubated with an unknown sample and the other half with a known sample (fertile semen), and following incubation the binding capacities of both samples are compared. In comparison to the HZA, the sperm zona pellucida binding assay uses the complete zona-enclosed oocyte. Low sperm binding to the zona pellucida may indicate spermatozoa issues related to the capacitation process (Burkman et al., 1990). Several investigations have tested these assays in order to predict male fertility with variable results. For example, a sperm-zona binding assay was used with cryopreserved ram and fresh boar semen (Berger et al., 1996) and in neither species was zona binding

correlated with *in vivo* fertility. However, the hemizona binding assay may be useful as a technique for evaluating semen quality in bulls (Fazeli et al., 1995), and stallions (Fazeli et al., 1995b). Furthermore, Oehninger et al. (1989) found that sperm samples with low fertility in IVF exhibited a lower number of sperm binding to the zona than samples with high fertilization rates (7.3 ± 1.4 vs. 62.1 ± 10.9, $P<0.05$, respectively). Confirming these results, Ardón et al. (2005) found that the number of accessory sperm in pigs (number of sperm found trapped in the zona pellucida) could be useful for sperm fertility evaluation. The hemizona-binding assay was developed and proven to be useful in better understanding the fertilization process in swine (Fazeli et al., 1995a). Fazeli et al. (1997), using a zona pellucida binding test, found that a high percentage of the sperm that initiate binding to the zona pellucida have an intact acrosome, and may be a clear indicator of why low fertility rates are obtained with cryopreserved semen. This information seems to indicate that the sperm zona pellucida binding test could be used to predict *in vitro* fertilization capacity and as a complementary test for assessing *in vivo* fertility.

2.3.2.3.2. Sperm penetration assay

The sperm penetration test, using zona-free hamster eggs is an *in vitro* test that assesses sperm functional competence or fertilizing ability. To conduct this assay it is necessary to recover the eggs from a hamster's fallopian tubes and place them into a hydrolytic enzyme solution to remove the cumulus mass and the zona pellucida. The zona-free oocytes are then incubated with a sperm sample of interest for 3 to 4 hours. The oocytes are then evaluated under a phase contrast microscope and the number of sperm with swollen heads (penetrated sperm) counted (Burkman et al., 1990). This test is useful for predicting the spermatozoa's potential to penetrate the oocyte. It is an indirect monitor of the sperm's ability to fuse with the egg membrane, and to undergo decondensation.

Several studies have shown a high correlation between penetration rate (zona-free hamster ova assay) and male fertility. For example, sperm from fertile boars have high penetration ability compared with the low penetration ability obtained from

sperm of infertile boars as demonstrated by Berger and Horton (1988) (93% vs. 11%, P<0.001); Berger and Parker (1989) (83% vs. 18%, P<0.001); Ivannova, and Mollova (1992) (66.03% vs. 23.08%, P<0.001). Martínez et al. (1998) using zona-free pig oocytes (homologous *in vitro* penetration assay (hIVP)) studied the relationship between the hIVP and boar fertility, concluding that this assay has a strong ability to identify fertile boars (sensitivity of 93.75%) and subfertile boars (specificity 83.33%). In all these studies, the sperm penetration rate shows that this assay may be useful in identifying subfertile males, but its ability to identify more subtle differences between fertile boars seems limited.

2.3.2.3.3. *In vitro* fertilization assay

In vitro fertilization (IVF) may be used to assess the spermatozoa's ability to fertilize a homologous zona-intact egg. In pig studies (see Xu et al., 1996ab; 1998) immature oocytes are usually collected from fresh ovaries from prepubertal animals and maturated *in vitro* (*in vitro* maturation; IVM). However, in order to simplify the IVF process, immature frozen pig oocytes (germinal vesicle stage) have been used to reduce the cost and time involved in the oocyte maturation process, as well as to reduce the variation in oocyte quality between rounds of IVF within the same experiment (Martínez et al., 1993). During IVF, the sperm sample is incubated with the immature or matured oocytes and, after a 12 to 18 h fertilization period, the oocytes are fixed, stained and examined under a microscope (Figure 2-9). Parameters evaluated following the IVM/IVF assay in the pig, include: penetration rate, polyspermy rate, monospermy rate, male pronuclear formation rate, number of sperm per oocyte and potential embryo production rate (Xu et al., 1998). This test evaluates the spermatozoa's potential to undergo several changes (biochemical and biophysical) such as capacitation, acrosome reaction, sperm-zona binding, sperm-oocyte binding and penetration and sperm decondensation, all of which are required for successful fertilization.

Previously, IVM and IVF techniques used for predicting boar fertility (Xu et al., 1996ab; Xu et al., 1998; Zhu et al., 2000) have been based on protocols established

for assessing oocyte quality and embryo production potential (Ding et al., 1992ab; Rath, 1992; Ding et al., 1994ab; Zak et al., 1997), which have inherently different objectives than the assessment of potential boar fertility. Differences in the *in vitro* and *in vivo* fertilization processes (female tract environment, time of ovulation and others) could be responsible for the low and medium correlations obtained (Xu et al., 1998). Furthermore, the variation between the different experiments using IVF techniques for assessing the boar makes it difficult to compare results. These variations can include: sperm fraction evaluated, sperm incubation time (capacitation and fertilization), manipulation of sperm, oocyte quality, use of mature or immature oocytes, final number of sperm per oocyte, etc. (Matas et al., 1994; Marchal et al., 2002; Flowers, 1997; Xu et al., 1998; Popwell and Flowers, 2004). Therefore, another key hypothesis explored in the experiments reported later in this thesis was that "*The standardization of the IVM-IVF technique, when used for semen evaluation purposes, could help to increase its correlation with in vivo fertility and provide a better understanding of sperm-oocyte interactions*".

From a practical perspective, although certain IVF parameters have demonstrated moderate correlations with in vivo boar fertility (oocyte penetration rates and number of sperm attached per oocyte, male pronuclear formation rate, potential embryo production rate) (Xu et al., 1998), this technique is expensive and time consuming and is likely to be impractical for routine semen evaluation in a commercial setting.

2.3.2.3.4. Sperm-oviduct binding assay

Recently, the *in vitro* evaluation of sperm's capacity to bind to the oviductal epithelium or oviductal cells has been used to understand sperm-oviductal interactions (Suarez, 1987; Hunter et al., 1991; Krishna, 1997; Smith and Nothnick, 1997; Fazeli et al., 2003; Holt et al., 2006), as well as to detect boar fertility differences (Petrunkina et at., 2001; Waberski et al., 2005). The sperm-oviduct explant assay uses fresh Fallopian tubes collected from gilts or sows. From these tissues, oviductal explants are prepared (Suarez et al., 1987; Petrunkina et al., 2001) and selected for cilia

integrity, and movement and stored at 4°C (>2h) until used. A sperm suspension is added to the explants and incubated for 15 min, then washed twice and evaluated using a videomicroscopic technique. Spermatozoa's capacity to bind to the oviduct epithelium has been associated with differences in the binding index between boars of different fertility, indicating that this test could be useful as a complementary test to assess boar fertility. As well, this assay might give valuable information on any changes in sperm during storage (Waberski et al., 2006). It is also important to note that the sperm population in the oviductal reservoir depends on the initial sperm quality (ejaculate and/or boar), the site of sperm deposition, number of sperm inseminated, and other factors, which may reflect ejaculate and/or male fertility differences.

2.4 CONCLUSIONS

In order to increase boar productivity and accelerate genetic improvement, the use of AI has increased tremendously over the last decade (Burke, 2000), thereby augmenting the demand for good quality processed semen. A major limitation in the optimization of boars for their use in AI programs has been the lack of good indicators of semen quality and boar fertility. Therefore, several studies have been conducted to develop practical laboratory tests for evaluation of semen quality.

In summary, spermatozoa undergo numerous changes before and during fertilization and there are several external factors (seminal plasma, semen collections, ovulation time, sperm transport to the female tract, type of AI, and others) that affect the fertilization process. As a result, it is not an easy task to develop an *in vitro* test that can be useful for assessing semen quality and predicting male fertility potential. Although several attempts have made to define "The test" that can be used for this purpose, results from only a few of these have been consistently correlated with *in vivo* and/or *in vitro* fertility (Berger and Parker, 1989; Flowers, 1997; Xu et al., 1998 Gadea and Matas, 1999). Therefore, due to the complexity of the fertilization process,

male fertility potential assessment requires the use of series of laboratory tests that not only address various aspects of the ejaculate (seminal plasma and sperm characteristics and functions), but take into consideration factors that affect the fertilization process (time of ovulation, insemination technique, number of AI doses, number of sperm per dose, etc). Ideally, male fertility would be evaluated by an economical, practical and rapid test, or tests, that could be used by AI centres and animal producers to assess semen quality and thereby increase the productivity of the farm. Unfortunately, such a test does not yet exist.

Collectively, improved knowledge of the fertilization process and of the techniques used for semen evaluation may provide us with a better understanding of the sperm characteristics that are important for successful fertilization and that will facilitate the development of adequate semen evaluation tests.

Based on early results (Xu et al., 1996ab; Xu et al., 1998) we hypothesized that the use of *in vitro* tests such as the IVM-IVF technique, that evaluate sperm characteristics directly related to the fertilization process, would provide meaningful information on the relationship between these *in vitro* measures of semen quality and differences in relative boar fertility.

Seconly, given the important role of seminal plasma, and particularly seminal plasma proteins, during the fertilization process, we hypothesized that fertility difference in boars that meet ejaculate laboratory standards could be related to different concentrations of specific seminal plasma proteins.

Thirtly, based on previous studies (Xu et al., 1996b; Juonala et al., 1998) we hypothesized that the different rates of motility loss when semen is extended and stored for a prolonged period of time (Days 3, 7 and 10) would reflect differences in relative fertility in vivo, representing a practical and economical boar fertility test that could be used at commercial level.

The compensatory effect of using high sperm numbers in AI doses (Tardif et al., 1999; Watson and Behan, 2002; Ardón et al., 2003) in order to determine relative boar fertility has been an obstacle to establish relationships between laboratory characteristics of an ejaculate and proven boar fertility. Therefore, the final hypothesis tested was that use of low sperm numbers per AI dose (1.5 billion sperm per AI dose) will reveal substantial differences in boar fertility *in vivo* and allow significant associations with *in vitro* semen assessments to be investigated.

Based on these underlying hypotheses, the following chapters describe a series of experiments using different laboratory techniques to assess semen quality and predict relative boar fertility. The results of these experiment are then used as the basis for discussing the development of practical and economical techniques that will have a significant impact on the swine industry.

Table 2-1. Cycle length, total duration of spermatogenesis, sperm transit time through the epididymis and the daily sperm production per gram of testis parenchyma (DSP) in different species (modified from Johnson et al., 1995, 2000; França et al., 2005).

Species	Cycle length (days)	Total duration of spermatogenesis (days) [a]	Sperm transit time through the epididymis (days)	DSP (millions)
Boar	8.6	38.7	9.0-11.8	24-27
	9.0	40.5	/	/
Wild boar	9.0	40.5	/	29
Bull	13.5	60.8	4.0-15.0	11-13
Buffalo	8.6	38.7	9.3	/
Ram	10.6	47.7	10.4-16.4	/
Goat	10.6	47.7	/	30
Stallion	12.2	54.9	4.9-13.8	16-21
Donkey	10.5	47.2	/	42
Rabbit	10.9	49.0	6.6-12.7	25
Dog	13.6	61.2	/	/
Cat	10.4	46.8	/	16
Rat	12.9	58.0	8.0-10.0	17-24
Mouse	8.6	38.7	5.0-5.8	47
Hamster	8.7	39.2	15.0-15.6	/
Monkey	9.5	42.7	10.5	/
Man	16.0	72.0	5.5	4-4.5

[a] Total duration of spermatogenesis is based on 4.5 cycles from A_1 spermatogonia.

Table 2-2. Low-Molecular weight components of mammalian seminal plasma (from Shivaji et al., 1990).

1. Ions	Sodium	Zinc
	Potasium	Iron
	Calcium	Chloride
	Magnesium	Bicarbonate
	Copper	
2. Organic acids	Ascorbic acid	Pyruvic acid
	Citric acid	Uric acid
	Lactic acid	
3. Sugars	Fructose	Galactose
	Fucose	Ribose
	Glucose	N-Acetylglucosamine
	Sorbitol	N-Acetylgalactosamine
	Sialic acid	D-ribose
	Mannose	Erythritol
	Manitol	Glycerol
4 Neutral lipids	Triglycerides	Cholesterol
	Diglycerides	Free fatty acids
5. Phospholipids	Phosphatidiylcholine	Phosphatidiylglycerol
	Phosphatidiylethanolamine	Cardiolipin
	Phosphatidiylserine	Sphingomyelin
	Phosphatidiylinositol	
6. Steroid Hormones	17-β Estradiol	Androstenedione
	Estriol	3-α-Androstenediol
	Estrone	Dehydroepiandrosterone
	Testosterone	Progesterone
	Dihydrotestosterone	
7. Prostaglandins	Prostaglandins E-1 (PGE-1)	Prostaglandins F-2
	Prostaglandins E-2 (PGE-2)	Prostaglandins A-1
	Prostaglandins E-3 (PGE-3)	Prostaglandins A-2
	17-OH Prostaglandin E-1	Prostaglandins B-1
	Prostaglandin F-1	Prostaglandins B-2
8. Amino acids	Aspartic acid	Tyrosine
	Threonine	Lysine
	Serine	Histidine
	Glutamic acid	Arginine
	Glycine	Tryptophan
	Alanine	Phenylalanine
	Valine	Hypotaurine
	Leucine	Methionine
	Isoleucine	
9. Polyamines	Spermine	Putrescine
	Spermide	
10. Nitrogenous Bases	Chlorine	Creatinine
	Phosphorylcholine	Carnitine
	Glycerylphosphorylcholine	Acetylcarnitine
	Acetylcholine	Serotonin
	Ergothioneine	Adrenaline
	Creatine	Noradrenaline
11. Miscellaneous	Ammonia	Sulfur
	Carbondioxide	Urea
	Cephalin	Vitamin B_{12}
	Plasmalogens	

Table 2-3. Major seminal plasma proteins associated with the fertilization process (modified from Zhu, 2000c)

Category	Name	Molecular weight (kDa)	Species observed	Source	Function	Reference
Spermadhesins	AWN-1	12-14	Porcine	ASG	Capacitation, zona pellucida binding,	Calvete et al., 1994 Liberda et al., 2006
	AWN-3	12-14	Porcine	ASG	Capacitation, sperm oviductal binding	Calvete et al., 1994
	AQN-1	12-14	Porcine	ASG	Capacitation	Calvete et al., 1994
	AQN-2	18-20	Porcine	ASG	Capacitation	Calvete et al., 1994
	AQN-3	12-14	Porcine	ASG	Cpacitation	Calvete et al., 1994
	hSA	14-18	Human			Kraus et al., 2005
	PSP-I BSP-A1 HSP-I	12-13 13 29-30	Porcine Bovine Stallion	Seminal Vesicles	Prevent premature AR and modify sperm membrane	Calvete et al., 1995; Rutherfurd et al., 1992; Zhu, 2000b Leblond et al., 1993 Romero et al., 1996
	PSP-II BSP-A2 HSP-II	13 13 29-30	Porcine Bovine Stallion	Seminal Vesicles	Prevent premature AR and modify sperm membrane, sperm-oviductal interaction	Calvete et al., 1995 Leblond et al., 1993; Gwathmey et al., 2003 Romero et al., 1996
	BSP-A3		Bovine		Modify sperm plasma membrane	Leblond et al., 1993
	BSP-30kDa		Bovine		Modify sperm plasma membrane	Leblond et al., 1993
Sperm-motility stimulating protein	Kallikrein (hK_2,hGK1)		Human	Prostate	Stimulate sperm motility	Deperthes et al., 1995
	AT-III		Porcine			Lee and Wei., 1994

Table 2-3b. Major Seminal Plasma Proteins associated with the fertilization process.

Category	Name	Molecular weight (kDa)	Species observed	Source	Function	Reference
Proteolitic activity	Kallikrein (hK2,hGK1)		Human	Prostate	Proteolitic activity on seminal coagulum	Deperhes et al., 1995
ZP-binding protein	ZPBP	16-23	Porcine		Regulate capacitation	Parry et al., 1992
	ACR-3	17	Porcine	ASG	Mediate the primary sperm-egg binding	Capcova and Peknicoca, 1997
Trypsin-like protein	Trypsin-like protein				Induce AR	Lee and Wei., 1994
Fertilization promoting peptide	FPP		Mammals	Prostate & Bulbo-urethral glands	Promote fertilization potential	Silvitier & Cockle, 1995; Kennedy et al., 1997
Protein C inhibitor	MCP(D46)	43	Human		Enhance fertilization	Seya et al., 1993
Sperm motility inhibitors	PCI		Human	Male Reprod tract Seminal vesicles	Inhibit Acrosin and other proteinase	Zheng et al., 1994; Robert & Gagnon, 1995
	SPMI	50	Porcine Human		Inhibit sperm motility	Iwamoto et al.,1992, 1995
Decapacitation factors	SPMIF	5.7	Porcine			Strzezék et al., 1992
	DCP	>100	Porcine		Inhibit AR	Bonilla et al., 1996
	Proteinase inhibitors				Inhibit AR and/or fertilization	Lee & Wei, 1994

AC Acrosome Reaction; ASG Assessory Sex Glands; AT Antitrombin; BSP Bovine Seminal Protein; DCP Decapacitation; EPP Fertilization Promoting Protein; HSP Horse Seminal Protein; MCP Membrane Cofactor Protein; PCI Protein C Inhibitor; PSP Porcine Seminal Protein; SMIF Sperm Motility Inhibiting Factor; SPMI Seminal Plasma Motility Inhibitor; ZPBP Zona Pellucida Binding Protein; ZP Zona Pellucida.

Table 2-4. Species differences in ejaculate characteristics (modified from Nalbandov, 1976; Prins, 1998; Senger, 2003)

Species	Normal volume of ejaculate (mL)	Normal density of ejaculate (10^6 sperm/mL)*	Range of pH
Cock[1]	0.2-1.5	50-6,000 (4,000)	6.3-7.8
Turkey	0.2-0.8	7,000 (7,000)	6.5-7.0
Boar	50-500	150-750 (300)	7.3-7.9
Bull	2-10	300-2,000 (1,000)	6.4-7.8
Ram[2]	0.7-2	2,000-5,000 (3,000)	5.8-7.3
Stallion[3]	30-300	30-8,000 (100)	6.2-7.8
Rabbit	0.6-6	100-2,000 (700)	6.6-7.5
Dog	2-14	1,000-9,000 (3,000)	6.7-6.8
Fox	0.2-4	30-300 (70)	6.2-6.4
Man	2-6	50-200 (100)	6.0-7.0**

*The most commonly observed values are in parenthesis.
**Wolters-Everhardt et al., 1986
[1]Great variations with management and strain.
[2]In breeding season (shortening day length)
[3]In breeding season (increasing day length)

Table 2-5. Relationships among sperm motility, sperm penetration rates, farrowing rates and number of pigs born alive for boar semen (from Flowers, 1997).

Motility[a] (%)	Sperm penetration rate [b] (%)	Farrowing rate [c] (%)	Number born alive
94.7	89.5[v]	86.9[v]	10.6[v]
	(58)	(460)	
82.3	81.7[vw]	87.1[v]	10.5[v]
	(55)	(330)	
76.1	84.3[vw]	84.5[v]	10.5[v]
	(50)	(300)	
66.2	74.7[w]	86.1[v]	10.1[v]
	(44)	(264)	
52.4	55.5[x]	72.4[w]	9.2[w]
	(40)	(201)	
44.2	34.7[y]	72.3[w]	9.2[w]
	(28)	(168)	
32.6	21.3[z]	51.7[x]	7.8[x]
	(17)	(85)	
SEM	4.8	5.8	0.3

*Motility is expressed as the average number of motile spermatozoa within the following classes: >90; 80-89; 70-79; 60-69; 50-59; 40-49; and 30-39.

[b]: Sperm penetration rate is defined as the percentage of eggs that were fertilized. The numbers in parentheses represent the number of ejaculates within a motility category.

[c:] Number in parentheses represent the number of sows inseminated within a motility category.

SEM: Pooled standard error of the mean.

[v,w,x,y,z:] Means within the same column with different superscripts are significantly different ($P<0.05$).

Figure 2-1. Schematic presentation of the journey of a spermatozoon through the male reproductive tract. 1) Seminiferous tubules; 2) rete testes; 3) efferent ducts; 4) caput epididymis; 5) corpus epididymis; 6) cauda epididymis; 7) deferent ducts; 8) seminal vesicle; 9) prostate gland 10) urethra; 11) bulbourethral gland; 12) bulb of penis (adapted from King, 1993; Shivaji et al., 1990).

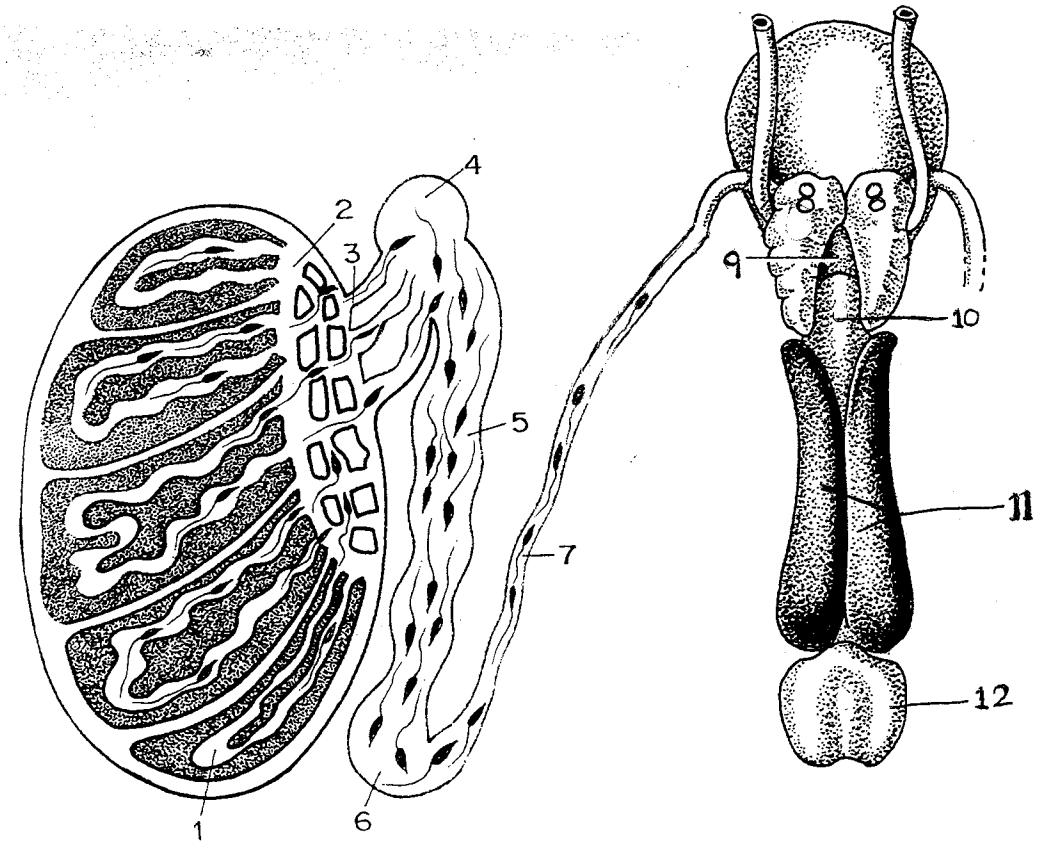


Figure 2-2. Schematic representation of normal sperm morphology (from Porterfield, 2001).

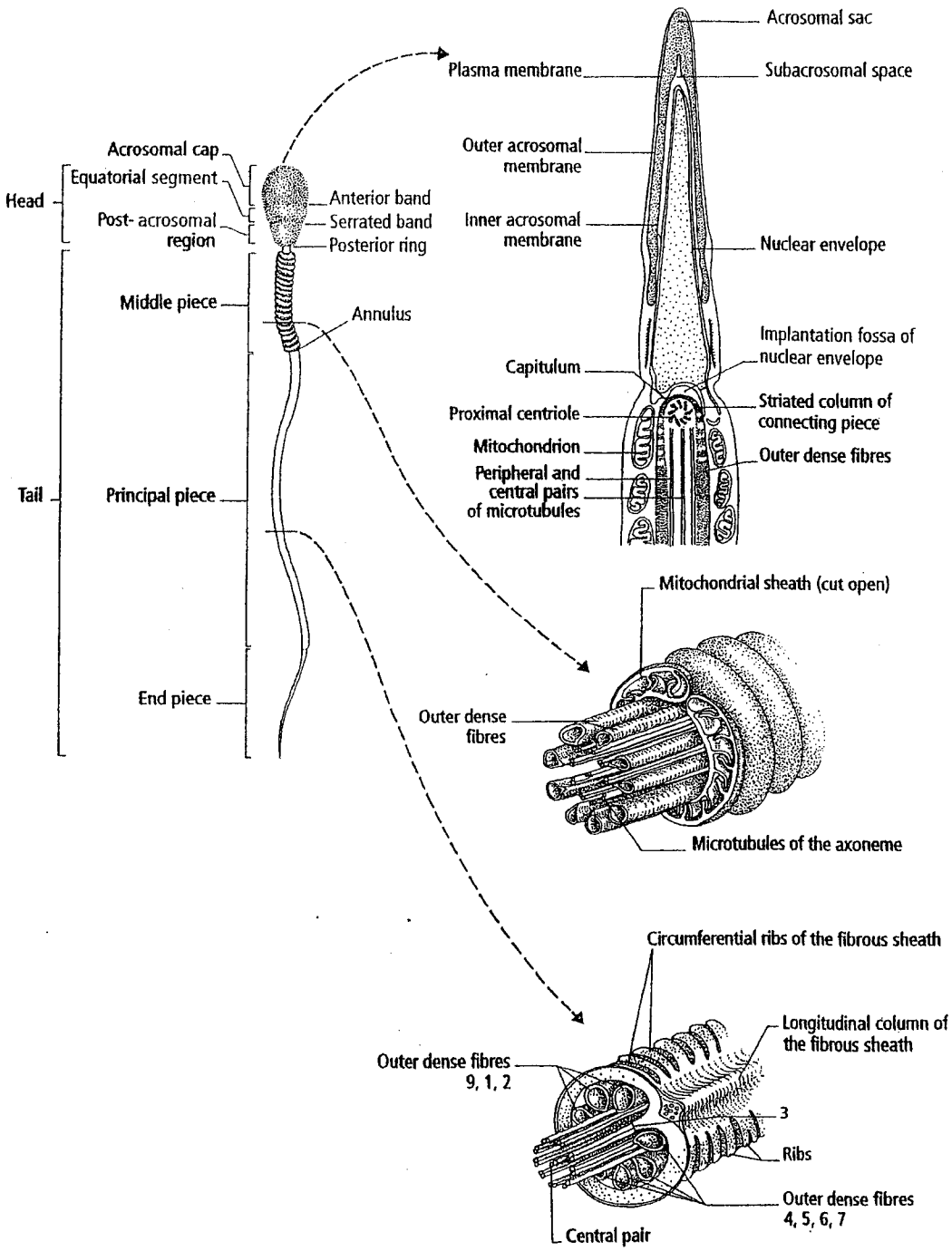


Figure 2- 3. Hormonal regulation of testicular function by hormones produced by the hypothalamus (Gonadotropin-releasing hormone (GnRH)), anterior lobe of the pituitary (Luteinising hormone (LH), Follicle-stimulating hormone (FSH)), the Sertoli cells (dihydrotestosterone (DHT), estrogens (E), inhibin, androgen-binding protein (ABP)) and the Leydig cells (testosterone (T)) (adapted from McLachlan et al., 1996; Seger, 1999; Porterfield, 2001).

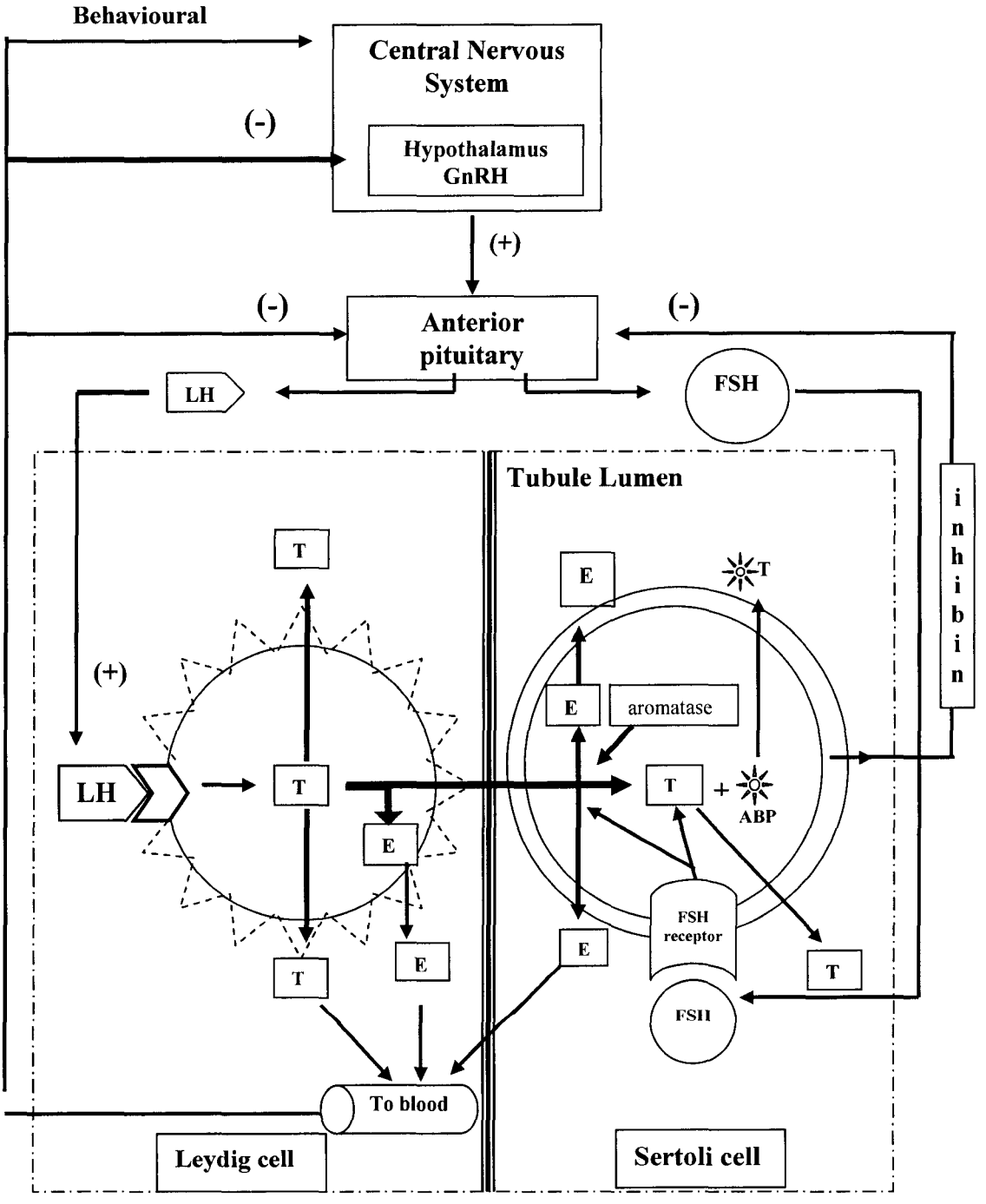


Figure 2- 4. Spermatogenesis (adapted from Porterfield, 2001).

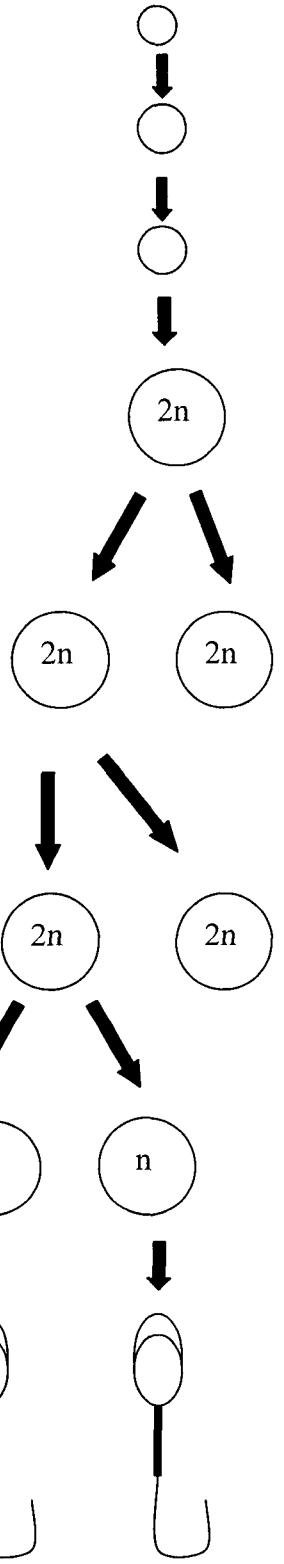
SPERMATOGENESIS

Undifferentiated A spermatogonia

Differenciated A spermatogonia (A_1 to A_4)

Intermediate Spermatogonia (In)

Spermatogonia B (spermatogonium) or stem cells (2n)



Mitotic division

Primary spermatocytes (2n)

1st meiotic division

Secondary spermatocytes (2n)

2nd meiotic division

SPERMIOGENESIS

Spermatids (n)

Sperm cells or spermatozoa (spermatozoon)

Figure 2- 5. Testis ductile system and its cell biology (modified from Porterfield, 2001; pictures from Senger, 1999)

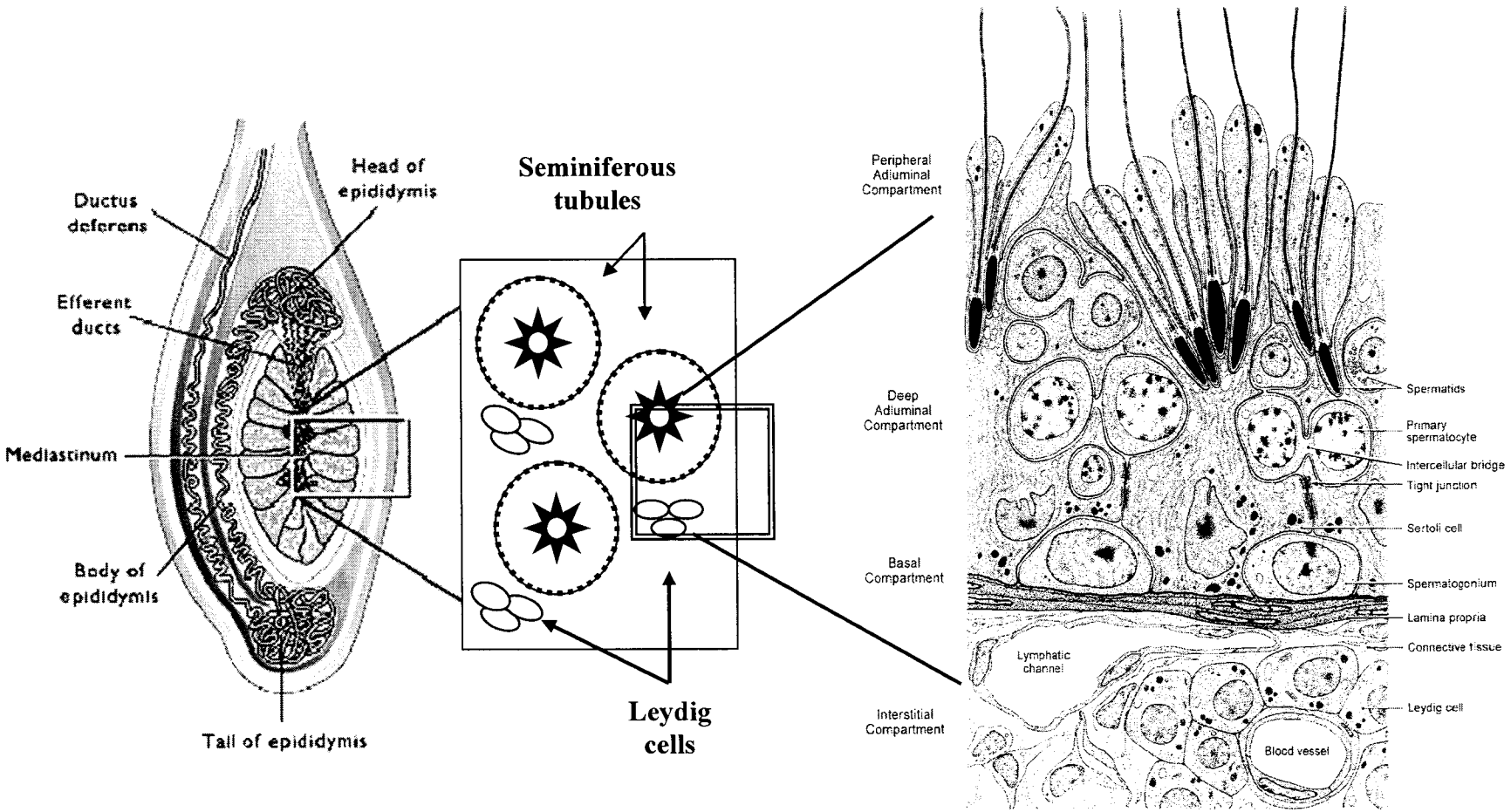


Figure 2- 6. Diagram of the cycle of rat spermatogenesis. During spermatogenesis the forming sperm cells undergo a progressive series of changes called stages which are grouped in cycles: each cycle is composed by 14 cell stages and they are represented by Roman numerals (from O'Donnell et al., 2006). Germ cell development is shown horizotally. A $_{(1-4)}$ type A $_{1-4}$ spermatogonia; In, intermediate espermatogonia; B, type B spermatogonia; PI, preleptotenespermatcyte; L, leptotene spermatocyte; Z, Zygotene spermatocyte; PS, pachytene spermatcyte; Di, diploid pachytene spermatocyte; II, secondary spermatocyte.

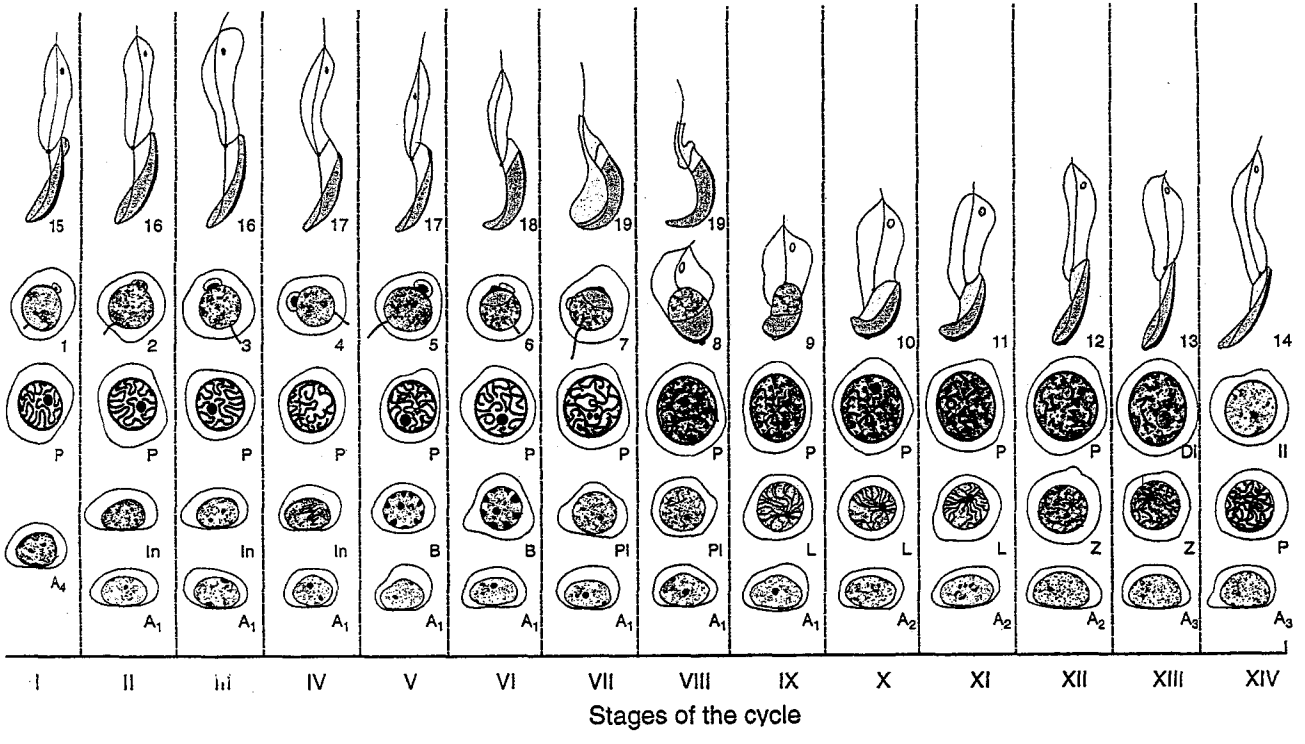


Figure 2- 7. Sperm capacitation signaling. Sperm capacitation is considered to initiate with the efflux of cholesterol from the plasma membrane that increases the influx of calcium (Ca^{2+}) and bicarbonate (HCO_3^-). Both ions activate adenylyl cyclase (AC), increasing cyclic-AMP which activates the protein kinase A (PKA) pathway and produces protein tyrosine phosphorylation. The activation of the EGF receptor and reactive oxygen species (ROS) receptors also produce protein phosphorylation. The increase of protein phosphorylation produces F-actin protein polymerization that enables the translocation of phospholipase C (PLC) to the plasma membrane that activates the PKC pathway. The increase of intracellular calcium also activates PKC pathways. During this process there is an efflux of ions such as K^+, Cl^- and Zn^{2+}, and an influx of Na^+ (adapted from Baldi et al., 2000).

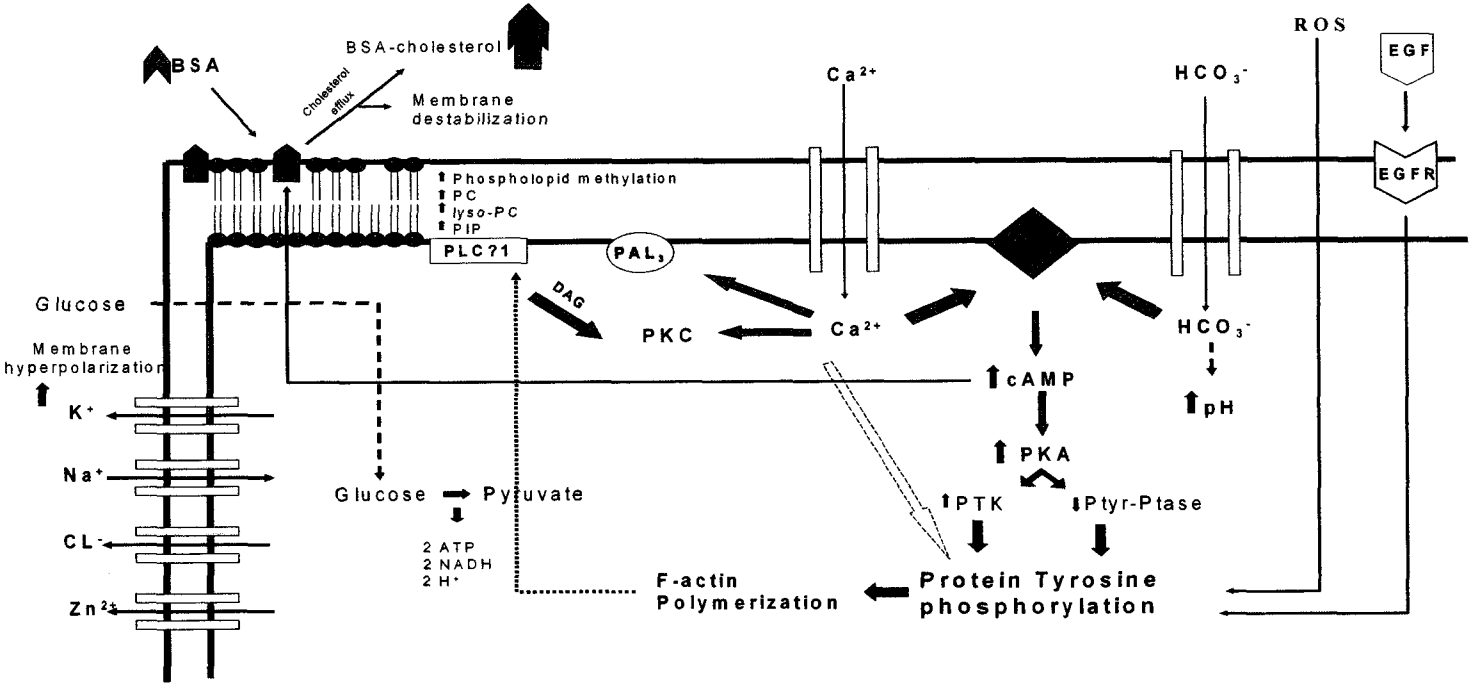


Figure 2- 8. Common types of abnormal spermatozoa. Sperm morphological defects categorized on the basis of sperm anatomical region (head, midpice and tail). A) Normal sperm; B) Sperm agglutination C) head, D) midpiece and E) tail. (Pictures from Senger, 2003; Ruiz-Sánchez et al., 2005; Rozeboom, 1999).

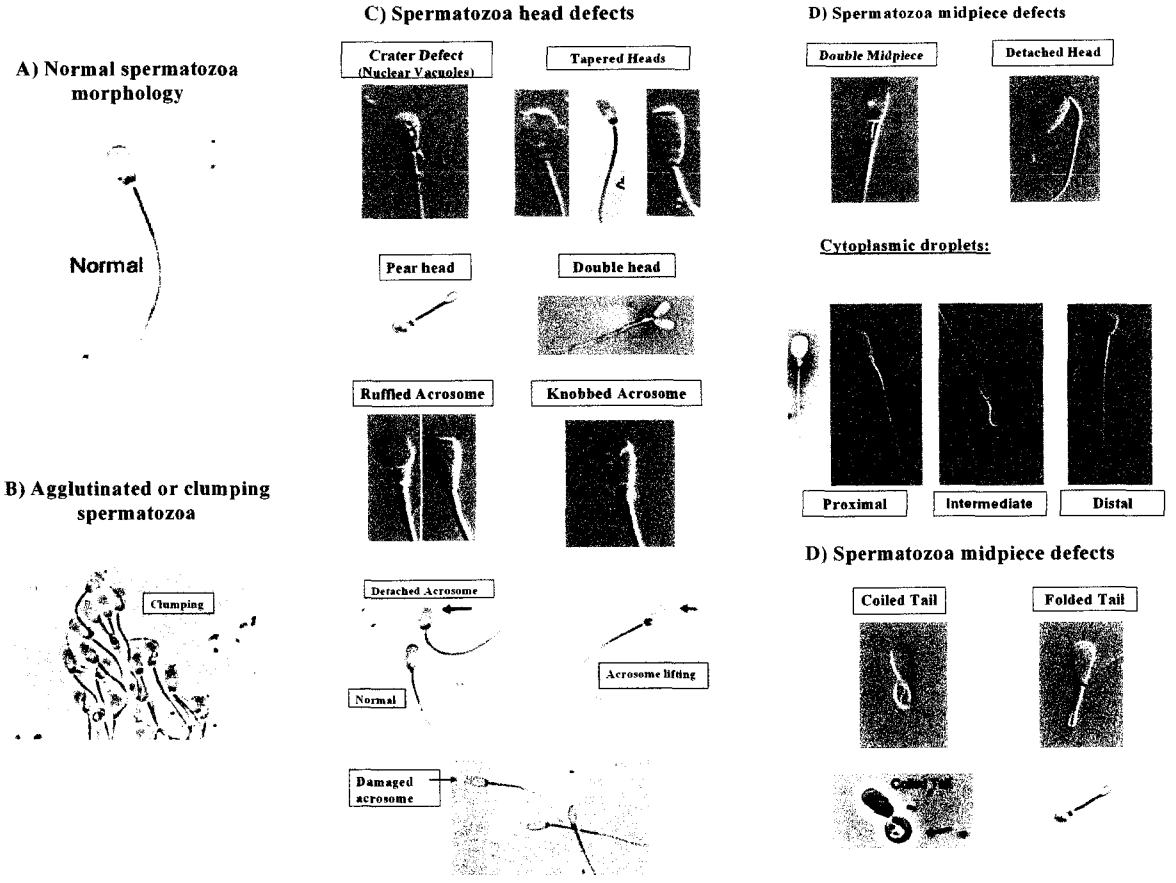
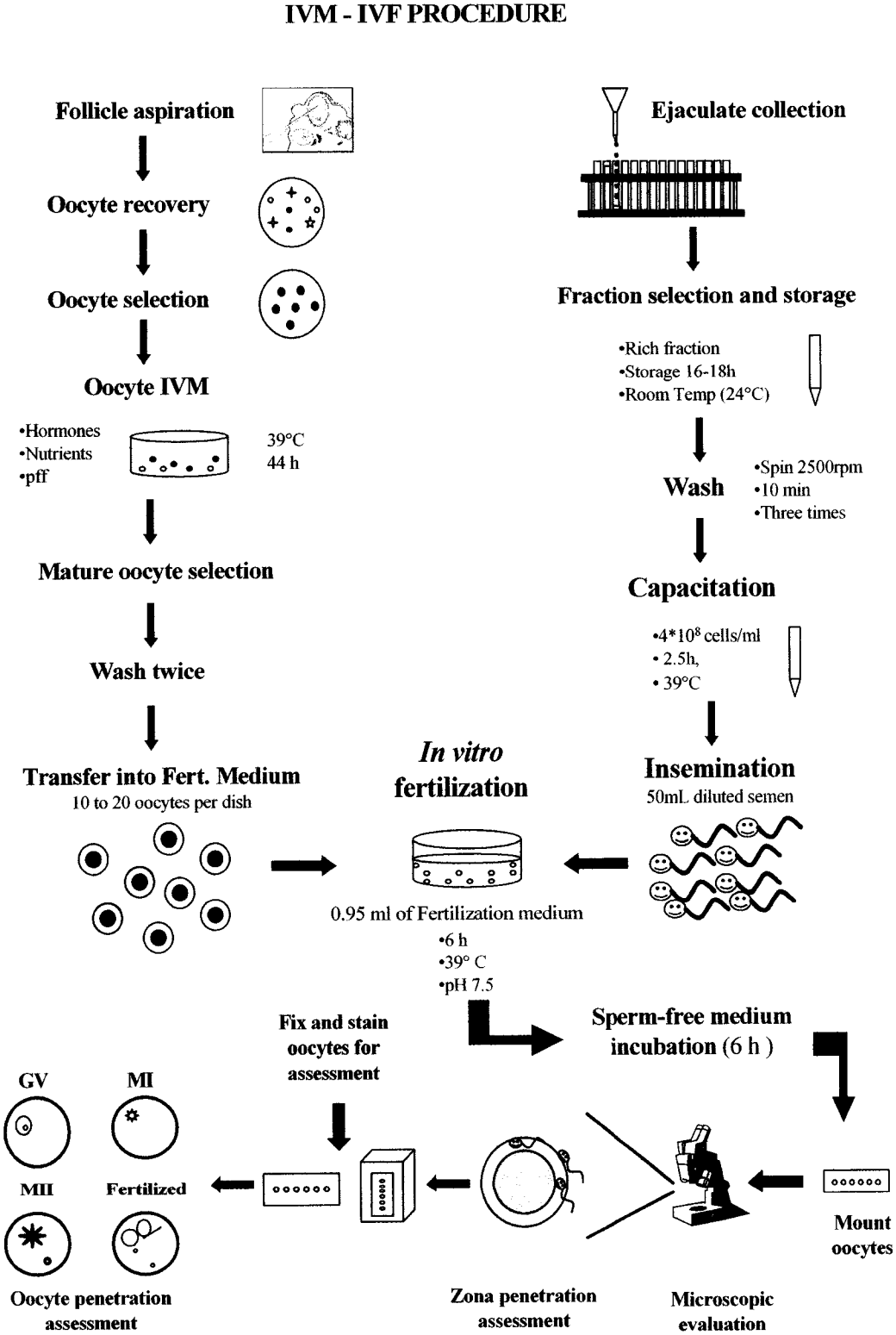


Figure 2- 9. Schematic representation of *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) procedures in pigs.



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CHAPTER THREE

THE PREDICTIVE VALUE OF ROUTINE SEMEN EVALUATION AND IVF TECHNOLOGY FOR DETERMINING RELATIVE BOAR FERTILITY[1]

3.1. INTRODUCTION

In the swine industry a single male has a more significant impact on efficiency and productivity than an individual female, and this impact is even higher with the use of artificial insemination (AI). In commercial AI centers, routine semen assessment generally includes the evaluation of ejaculate characteristics such as sperm concentration, morphology, viability, and motility. Although some of these characteristics can be used to detect male reproductive disorders that result in low fertility, they are not useful for predicting relative fertility in healthy boars with ejaculate quality that meets normal industry standards (>70 % motility and <30% abnormal sperm), even though the productivity of these boars may still be substantially different. Due to the complexity of the fertilization process, several sperm attributes are required for successful fertilization, such as the ability to undergo capacitation, hyperactivation, the acrosome reaction, binding to the zona pellucida (ZP) and oocyte penetration. A range of *in vitro* tests have been used to evaluate sperm characteristics directly related to the fertilization process, including the hypoosmotic-swelling test (Vázquez et al., 1997; Chun-Xia et al., 2000; Pérez-Llano et al., 2001), sperm-ZP binding test (Fazeli et al., 1995; Berger et al., 1996), and the *in vitro* fertilization (IVF) of homologous zona-intact, *in vitro* matured (IVM) oocytes (Xu et al., 1998). However, only ZP penetration rate (Ivannova and Mollova, 1993) and oocyte penetration rate (Berger and Parker 1989; Gadea et al., 1998; Gadea and Matas, 2000) have been successfully used to identify fertile vs. subfertile boars and/or

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ejaculates. It is unclear whether these techniques would be effective for predicting the relative fertility of boars with ejaculates that meet normal laboratory criteria for extension and use for AI. Standardized IVM and IVF techniques have previously been used in our laboratory to assess boar semen quality (Xu et al., 1996a,b) and Xu et al. (1998) reported that the estimated potential embryo production rate (an integrated measure of sperm quality *in vitro*), and number of sperm attached per oocyte, accounted for up to 53% of the variation in litter size, when 2 billion sperm per AI dose were used to determine relative boar fertility *in vivo*. The importance of using low sperm doses for AI to initially determine relative boar fertility *in vivo* has been confirmed in a number of subsequent studies (Tardif et al., 1999; Watson and Behan, 2002; Ardón et al., 2003). This approach likely avoids the compensatory effect of using high sperm numbers per dose for AI and was one of the concepts incorporated into the study described below.

Previous studies in our group (Xu et al., 1996b) reported inter-boar differences in the motility of extended semen at day 7, and found a high correlation ($r=0.8985$, $P=0.0001$) between these characteristics and penetration rate *in vitro*. Furthermore, Juonala et al. (1998) found positive correlations with *in vivo* fertility and diluted stored semen. Therefore, evaluation of motility of extended semen at different days of storage represents an inexpensive indicator of boar fertility *in vivo*. We, therefore, hypothesized that the different rates of motility loss of extended stored semen would reveal difference among boars that could be a reflection of their relative fertility *in vivo*, representing a practical technique for assessing semen quality and for predicting boar fertility.

The principal objectives of the present study was to further evaluate effective predictors of relative boar fertility using; 1) a population of boars that would be considered acceptable for use in AI programs on the basis of ejaculate/sperm characteristics measured in most commercial AI centers, 2) only 1.5 billion sperm per AI dose to make comparisons of relative boar fertility *in vivo*, and 3) estimates of declining motility in extended and stored semen doses prepared for AI. These results

were also compared with data from IVF technology using homologous, zona-intact, *in vitro* matured pig oocytes.

3.2. MATERIALS AND METHODS

3.2.1. Boar fertility evaluation in vivo

This experiment was conducted at the Swine Research and Technology Center of the University of Alberta, according to the guidelines of Canadian Council for Animal Care and with approval from the Faculty Animal Policy and Welfare Committee.

A total of nine Genex Large White boars were received at 26 to 28 wk of age (Genex Swine Group, Regina, Saskatchewan, Canada), evaluated in three groups of three boars each, and identified by color and group (Blue-1, Red-1, Green-1; Red-2, Green-2, Yellow-2; Blue-3, Red-3 and Purple-3). After adapting and training each group of boars for a period of 3 to 4 wk, semen collections were standardized to twice per week using the gloved hand technique. A complete single ejaculate was collected into sterile pre-warmed 15-mL Falcon tubes (VWR Canlab, Mississauga, Ontario) as described by Xu et al. (1996b). Tubes containing the first Sperm-Rich fraction were visually identified: The concentration of the last tubes of the first Sperm-Rich fraction with a concentration of $\geq 100 \times 10^6$ spermatozoa per mL were then confirmed using a calibrated colorimeter (model 254 Sherwood Scientific Ltd. Cambridge, UK) and this and the previous tubes were included as part of the first Sperm-Rich (SR) fraction. The tubes containing less than 100×10^6 spermatozoa per mL were considered to be part of the Sperm-Free fraction (SF) and discarded with any subsequent Sperm-Rich fractions collected from the same ejaculate (Figure 3-1). All the tubes from the first Sperm-Rich fraction were then combined in a pre-warmed thermos by filtering through gauze to eliminate any gel component, to create the complete first SR fraction

for routine semen evaluation (twice a week during the evaluation period) and breeding (every three weeks out of four).

Semen evaluations were performed by two well-trained and experienced individuals. Preliminary studies were performed to ensure that morphology and motility scores obtained by these individuals were comparable. The SR fraction was processed as follows:

1) Total volume (mL) was measured by weighing the SR fraction using an electronic balance and assuming a density of 1 g per mL.

2) Sperm morphology was evaluated using an eosin-nigrosin vital stain (Minitube of America, Inc. Verona, WI) under a microscope (Olympus CH30, Japan) fitted with a x 40 phase contrast lens. A smear was prepared by mixing a drop of semen with a drop of stain on a preheated glass slide. The smear was air-dried and at least 100 sperm were evaluated and the percentage of spermatozoa with abnormal heads, cytoplasmic droplets (both distal and proximal), abnormal tails and total of abnormal sperm were calculated. If a high proportion of detached heads were found, a second smear was prepared in order to confirm the results.

3) Sperm concentration was measured using the calibrated colorimeter mentioned above. To ensure accurate sperm counts, calibration was checked every 2 to 3 wk using a haemocytometer chamber. Also, when the concentration of the ejaculates exceeded the critical point, samples were re-diluted and measured again to confirm ejaculate concentration. Periodically, sperm concentration from extended semen was verified using the haemocytometer chamber. Evaluation of optimum performance of the colorimeter used for sperm concentration measurements was determined by a pilot study presented in Appendix 4, Figure A-1.

4) Progressive motility on the day of collection (Day 0) was evaluated at 37°C using the same microscope with a final magnification of x 400, screening at least 5 different fields. The estimate for progressive motility was subjectively scored to the

nearest 5%. Diluted semen was stored at 17°C in aliquots of 3 mL (5 mL glass tubes) for further motility evaluations. On Days 3, 7, and 10 a sample of diluted semen (3 mL aliquot) was gently mixed and warmed up at 37°C for 20 min. Caffeine was added to the sample prior to examination and motility assessment was carried out following the same protocol used above.

5) Finally, on breeding weeks, the SR fraction was diluted with Beltsville Thawing Solution containing antibiotics (BTS, Minitube of America, Inc. Verona, WI) to 1.5 x 10^9 morphologically normal, motile sperm per 50-mL dose (calculated as the average of motile sperm (%) and normal sperm (%) minus 100, giving the proportion of additional sperm per AI dose).

Diluted semen from each boar was identified by color (Minitube of America, Inc. Verona, WI) and used to breed approximately equal numbers of gilts over the same breeding week. The purpose of using relatively low sperm numbers per dose was to improve the ability to detect differences in proven fertility *in vivo*. Extended semen was stored at 17°C and gently agitated once a day until the time of insemination. Each group of boars was evaluated during a 6.5 ± 1 mo period (from May to February) and semen from each boar was used to breed 50 ± 5 gilts during this time, with breeding occurring three weeks out of four depending on gilt availability.

Genex hybrid gilts (Genex Swine Group, Regina, Saskatchewan, Canada) were housed in individual stalls and fed to appetite with a standard gestation (dry sow) diet. All gilts were bred at second or third estrus. Estrus was synchronized during the previous estrous cycle with the oral progestagen, Altrenogest (Regumate, Hoechst-Roussel Vet., Regina, Saskatchewan, Canada), ensuring that the majority of gilts were bred with semen no older than three days. Gilts were checked twice daily (0700 and 1900) for standing estrus using the back pressure test during fence line contact with a mature boar, and inseminated with semen from the same test boar 24 h after first detection of standing heat and again 12 h later if the animal still displayed a strong standing reflex. Quality of insemination and duration of standing heat were recorded

for all gilts. Test boars were collected Tuesday morning and Friday afternoon. The majority of breedings (95%) were completed from Tuesday to Friday. Animals with poor standing heat and/or inseminations were removed from the experiment. Pregnancy was confirmed by ultrasonography at day 30. Pregnancy rate (% of bred animals pregnant at day 30), farrowing rate (% of bred animals that farrowed) and total litter size (total number of piglets born alive or dead in a litter) were recorded. A boar Fertility Index (FI) was also calculated as the total piglets born divided by the number of gilts initially bred per boar. Both gilts and boars were kept under controlled conditions (temperature and hours of light) in order to reduce any seasonal effect.

3.2.2. Semen evaluation in vitro

Semen quality from each boar was also evaluated using an oocyte *in vitro* maturation and fertilization procedure described previously (Xu et al., 1996ab) with substantial modifications described below.

3.2.2.1. Culture media

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis. MO, USA). Oocyte Maturation Medium consisted of modified M199 (M199 (Gibco, Grand Island, NY, USA), supplemented with 861 μg/mL glucose; 155 μg/mL sodium pyruvate; 155 μg/mL polyvinyl alcohol); 70 μg/mL cysteine, 70 μg/mL L-ascorbic acid, 35 μg/mL insulin (I1882) 2.5 μg/mL pLH-BIO (AFP-12389A, NIDDK, Torrance, California, USA), 2.5 μg/mL pFSH-BIO (AFP-9400D, NIDDK Torrance, California, USA), 10 ng/mL EGF. The maturation medium was also supplemented with 10% (v/v) of porcine follicular fluid (pFF) obtained from clear follicles 5-8 mm diameter, centrifuged at 1680 x g for 30 min, filtered with a 0.22 μm syringe filter and stored at –15°C until use.

Fertilization Medium was prepared using a modified TRIS solution (2.30 mg/mL TRIS, 6.28 mg/mL sodium chloride, 0.21 mg/mL potassium chloride, 0.52

mg/mL glucose, 0.52 mg/mL sodium pyruvate, and 1.05 mg/mL calcium chloride), supplemented with 1.04 mg/mL caffeine/sodium benzoate and 3.80 mg/mL BSA (A7888).

Sperm-Free Medium consisted of North Carolina State University (NCSU) 23 medium (Peters and Wells, 1993) containing 75 μg/mL penicillin, 50 μg/mL streptomycin and supplemented with 4 mg/mL BSA (A8022) immediately before use.

3.2.2.2. Semen collection

At least nine times during the boar evaluation period, ejaculates were collected into sterile pre-warmed 15-mL Falcon tubes and 5 mL aliquots of the first Sperm-Peak (SP) fraction were used for *in vitro* assessment. The SP fraction was defined as the tube containing the highest sperm concentration within the first SR fraction. The SP fraction was initially determined visually as the tube with the greatest density and confirmed colorimetrically by comparing the sperm concentration of this tube to the next tube collected. The remainder of the SP fraction and the other tubes in the SR fraction were combined and used for routine semen evaluation and AI as described in section 3.2.1 (Figure 3-1).

3.2.2.3. Oocyte collection and *in vitro* maturation

Ovaries with well-developed Graafian follicles were routinely collected from prepubertal gilts immediately after slaughter through the cooperation of staff of a local abattoir (Olymel, Red Deer, Alberta, Canada). To minimize temperature changes and bacterial contamination, the ovaries were transported to the laboratory within 90 min of collection in thermos flasks containing sterile physiological saline at 30°C with added antibiotics (75 μg/mL of penicillin G potassium; 50 μg/mL of streptomycin sulphate). Ovaries were then washed three to four times with pre-warmed saline solution before aspiration of follicles. Modifications to established oocyte collection and *in vitro* maturation procedures were based on information provided by Dr. B. Day (Department of Animal Science, University of Missouri-Columbia, Columbia, Missouri) as follows: Cumulus-oocyte-complexes (COC's) were aspirated from clear

follicles with a diameter of 3 to 6 mm using a 10 mL syringe fitted with an 18 ga needle. COC's were washed twice with PBS and then once with PBS containing 1% (v/v) of pFF (PBS-pFF). Only COC's with a uniformly dark cytoplasm and with several compact layers of cumulus cells were selected and washed twice with PBS-pFF, and then washed twice more with Oocyte Maturation Medium. Approximately 40 COC's were then transferred to a Falcon culture dish (Becton Dickinson Labware, USA) containing 2 mL of Oocyte Maturation Medium, and incubated for 40 to 44 h at 39°C in an atmosphere of 5% (v/v) CO_2 in air.

3.2.2.4. Sperm capacitation

Freshly ejaculated samples representing the SP fraction of the ejaculate were kept at room temperature (20°C) for approximately 16 h after collection. A 2 mL aliquot of the sperm sample was then transferred into a 15 mL Falcon tube, and diluted with 5 mL Sperm Washing Medium (PBS supplemented with 0.1% BSA (w/v)) and washed by centrifugation for 5 min at 1000 x g. The supernatant was discarded and the sperm pellet resuspended in sperm washing medium. This washing procedure was repeated twice. The supernatant was then discarded and the sperm pellet resuspended at 4 x 10^8 sperm/mL in Fertilization Medium and incubated at 39°C under an atmosphere of 5% (v/v) CO_2 in air for 150 min. Our own serendipitous observations, subsequently confirmed by observations in other laboratories (personal communication from Dr. B. Day, Department of Animal Science, University of Missouri-Columbia, Missouri), led us to increase the time of sperm incubation from 90 min to 150 min to substantiality increase the oocyte penetration rate *in vitro*.

3.2.2.5. *In vitro* fertilization

After *in vitro* maturation, COC's with fully expanded cumulus cells were selected and washed twice in Fertilization Medium. Ten oocytes were transferred to each well of a 4-well culture dish (Nucleon 176740, Mississauga, ON) containing 0.95 mL of Fertilization Medium per well and incubated pending *in vitro* fertilization.

After capacitation, sperm motility was evaluated and the sperm concentration adjusted to produce a final ratio of 5 x 10^4 motile sperm per oocyte. Sperm and oocytes were incubated at 39°C under 5% (v/v) CO_2 in air for 6 h. After fertilization the oocytes were washed three times with Sperm-Free Medium. They were then transferred into a 4-well culture dish containing Sperm-Free Medium and cultured for a further 6 h ± 30 min at 39°C under 5% (v/v) CO_2 in air. Finally, 12 h after fertilization, the oocytes were transferred from the incubator to a refrigerator at approximately 5°C for 10 ± 2 h until examination.

3.2.2.6. Sperm penetration assessment

Zona pellucida penetration was examined by initially mounting the unstained oocytes on slides, evaluating them using a phase-contrast microscope (Dialux 20 EB Leitz Wetzlar, Germany) at a magnification of x 200 and x 400, and recording both the zona pellucida penetration rate (percentage of oocytes with sperm within the zona pellucida) and the number of sperm penetrating the zona (all sperm with both head and tail within the zona pellucida viewed in the saggital focal plane). The oocytes were then fixed for at least 48 h in slide fixing solution (1:3 acetic acid: ethanol), stained with lacmoid, and examined 1 d later using a phase-contrast microscope at magnifications of x 200 and x 400 for penetration rate (percent of mature oocytes penetrated), number of sperm per oocyte (average number of penetrated sperm, from slightly swollen sperm head(s) to male pronuclei, per oocyte), monospermy rate, polyspermy rate, male pronuclear formation rate (MPN-f; estimated as the percentage of penetrated oocytes with at least one male pronucleus), potential embryo production rate (% of penetrated oocytes with both a female and a single male pronuclei), and the percentage of penetrated oocytes with zero (zeroMPN), one (oneMPN), and more than one male pronucleus (>1MPN).

3.2.3. Statistical analysis

A total of 15 to 16 groups of gilts were bred during the evaluation period. To increase the number of gilts bred and farrowed per boar during specific breeding periods that were subsequently used to correlate *in vivo* estimates of boar fertility to

laboratory and *in vitro* assessment of semen quality, data were analyzed at 7 time intervals (boar age), representing successive periods from the start of breeding (time 1; 29-32 wk old) to the last evaluation period (time 7; 64-68 wk old). The results of *in vitro* fertilization and routine semen evaluation were grouped on the same basis for the statistical analysis.

Differences among boars (n=9) for total litter size, routine semen evaluation and IVF characteristics were analyzed as a repeated measures analysis using a mixed procedure of Statistical Analysis System (SAS version 8.2, SAS Institute Inc. Cary, NC, USA). The fixed effects were time (7 levels), boars (n=9), and their interaction, and the boar group as a random effect. In all statistical models, the Kenward-Roger option was used to calculate the denominator degrees of freedom. The variance-covariance matrix was chosen for each statistical model by an interactive process wherein the best fitting model was based on Schwarz's Bayesian criteria. Least square means and standard errors were generated and separated using a pdiff adjusted by Tukey option for significant, fixed effects. All IVF percentile data were subjected to arcsine transformation before analysis. All data are presented as LSmean ± standard error of LSmeans.

As the IVF results from the second breeding time interval for one group of three boars were inadvertently lost due to technical problems, data from this time interval were removed for all the boars, in order to be able to run the statistical program, leaving data from six time periods per boar for analysis of *in vitro* differences. Differences among boars for conception rate and farrowing rate were evaluated by Chi-square analysis using boar and time as independent variables.

A Stepwise linear regression model was used to determine the association between *in vivo* characteristics (conception rate, farrowing rate, total litter size, and Fertility Index) and *in vitro* fertilization variables, and between *in vivo* characteristics and routine semen evaluation characteristics (SAS version 8.2, SAS Institute Inc. Cary., NC, USA). Associations that were significant at the 0.15 level, were then

included in the regression model. Independent association between selected variables (IVF and routine semen evaluation variables) and *in vivo* fertility were evaluated using INSIGHT procedure (SAS, 2003).

Data from 38 gilts that were identified as having problems during artificial insemination were removed in order to reduce the effect of mis-breeding on estimates of boar fertility *in vivo*. Data from the last group of gilts (11 gilts) bred with the first group of boars that were identified with problems related to an outbreak of circovirus disease were also removed from the analysis.

3.3. RESULTS

3.3.1. Fertility evaluation in vivo

The *in vivo* performance of boars is show in Tables 3-1 and 3-2. There were differences among boars in pregnancy rate and farrowing rate but no effect of time (boar age) for these characteristics. For pregnancy rate and farrowing rate, eight boars showed superior fertility, among which boar R-2 and B-1 demonstrated the highest fertility and were significantly superior to boars R-1 and G-1 in terms of pregnancy rate. Boar G-1 had reduced pregnancy and farrowing rates compared to all other boars studied, except R-1. There were significant effects of both boar and time on total litter size (Table 3-1 and 3-2), but no boar by time interaction (P=0.88). Boars R-2 and Y-2 had a higher litter size born than boars G-1 and B-3. Boar G-1 produced the lowest litter size born but was not different to five other boars. There were significant differences between boars in Fertility Index, with R-2 having the highest, and R-1 and G1 the lowest Fertility Index. The Fertility Index was also affected by time. Across boars, pregnancy rate and farrowing rate were highly correlated ($r=0.95$; $P<0.0001$). There were much weaker, albeit highly significant, correlations between both pregnancy rate and farrowing rate, and total litter size ($r=0.42$; $P<0.0005$, and $r=0.44$; $P<0.0004$, respectively).

3.3.2 Routine optical evaluation of semen quality

There were differences (P<0.0001) among the nine boars in total sperm number per ejaculate, the percentage of progressively motile spermatozoa in extended semen on Days 3, 7, and 10, and in the percentage of sperm with cytoplasmic droplets (Table 3-3), but no effect of time (boar age) was found for these characteristics. As shown in Table 3-3, sperm motility in diluted semen on Day 3 to Day 10 in boars R-1 and G-1 was consistently lower than other boars. The percentage of detached heads increased with age (P=0.010) but there was no boar effect (data not shown). Significant interactions between boar and time (boar age) were observed for the total volume of ejaculate, sperm numbers (concentration) per mL, percentage of progressively motile spermatozoa in raw and extended semen on Day 0, percentage of morphological normal sperm (Table 3-4), and the percentage of bent tails in fresh semen (data not shown). Boars G-1 and R-1 presented the lowest values for percent normal sperm and were inconsistent over time (Table 3-4).

3.3.3. In vitro fertilization data

Boar affected all measures of semen quality evaluated *in vitro* (Table 3-5), whereas only oocyte penetration rate (P=0.002), polyspermy rate (P=0.001), potential embryo formation rate (P<0.001), and oneMPN (P<0.001) were different over time (boar age) (data not shown). Significant interactions between boar and time (boar age) were observed for monospermy rate and zeroMPN.

3.3.4. Correlations relating boar fertility to routine semen evaluation and IVF variables

Because the primary objective of this study was to identify semen characteristics predictive of relative boar fertility, and because *in vivo* measures of boar fertility did not change over time, only those variables measured with IVF and routine laboratory techniques that showed differences among boars, but no differences over time (boar age), or a boar by time interaction, were included in the stepwise linear regression analyses of relationships to *in vivo* fertility.

On this basis, the routine laboratory variables used to determine correlations to pregnancy rate, farrowing rate, total litter size and Fertility Index were; the percentage of sperm with cytoplasmic droplets (cd) and the percentage of progressively motile spermatozoa in extended semen on Days 3, 7, and 10. Correlation coefficients of the multiple linear regression analyses from selected variables are presented in Table 3-6. The regression equations obtained were as follows:

Pregnancy rate = 61.75 + 0.52 (Motility Day 10); r^2=0.12 (P=0.0060)
Farrowing rate = 44.21–5.47(cd) + 0.72 (Motility Day 7); r^2=0.17 (P=0.0006)
Total liter size = 4.95 – 0.71 (cd) + 0.10 (Motility Day 7); r^2=0.22 (P=0.0040)
Fertility index = 0.34 – 1.21 (cd) + 0.15 (Motility Day 7); r^2=0.27 (P<0.0001)

Similarly, the IVF variables tested for correlations to pregnancy rate, farrowing rate, total litter size and Fertility Index were: average number of sperm penetrated the zona pellucida, average number of male pronuclei (aveMPN), average number of sperm penetrated per oocyte, MPN-f and >1MPN rates. Correlation coefficients of the multiple linear regression analyses from selected variables are presented in Table 3-7. The regression equations obtained were as follows:

Pregnancy rate = 73.5 + 23.8 (MPNformation); r^2=0.16 (P=0.001)
Farrowing rate = 70.4 + 23.8 (MPNformation); r^2=0.17 (P=0.001)
Total litter size = 9.7 + 0.7 (aveMPN); r^2=0.09 (P=0.010)
Fertility Index = 6.9 + 3.6 (MPNformation); r^2=0.12 (P=0.006)

Multiple linear regressions including all IVF and routine semen evaluations variables are presented in Table 3-8. As well independent relationships between selected variables and *in vivo* fertility parameters are summarized in Table 3-9.

3.4. DISCUSSION

Based on the earlier study of Xu et al. (1998), it appears that relationships can be established between *in vitro* measures of semen quality and differences in relative boar fertility, even when ejaculate/sperm quality meets standard industry criteria for AI use. Therefore, it is important to emphasize that compared with other published studies of ejaculate quality (Berger and Parker, 1989; Gadea et al., 1998; Gadea and Matas, 2000), all the boars used in the present study exceeded normal industry standards (>80% progressive motility and >85% morphologically normal sperm).

Several attempts have been made to develop effective techniques for assessing semen quality and predicting male fertility. As discussed earlier, few measures of sperm attributes have been correlated with *in vivo* and/or *in vitro* fertility (Xu et al., 1998; Berger and Parker, 1989; Gadea et al., 1998; Gadea and Matas, 2000; Flowers, 1997). In earlier studies, an obstacle to establishing relationships between laboratory characteristics of an ejaculate and proven boar fertility may be due to the use of high sperm doses for AI, which may partially compensate for differences in fertility among boars. By using only 1.5 billion morphologically normal, and motile, sperm per AI dose in the present study, we were able to demonstrate substantial differences in boar fertility *in vivo*.

A difference of more than 15% in farrowing rate and over 2 total pigs born per litter between the most and least fertile boars suggests that an ability to predict and exclude boars performing such as G-1 and R-1 in the present study, would have a considerable economic impact on production efficiency. It is possible that increased sperm numbers per AI dose could have partially offset the lower fertility of boars G-1 and R-1. However, based on unpublished data, Dr. W. Flowers (personal communication, Department of Animal Science, College of Agriculture and Life Sciences. North Carolina State University, North Carolina, USA) suggested that only partial compensation can be achieved by increasing sperm numbers. Furthermore, observed differences in pregnancy rate and farrowing rate in the present study were

consistently different among boars over the period of analysis. These data suggest that results obtained from evaluation of semen collected immediately after the boars have been trained for AI use will be predictive of subsequent relative fertility. Also, as pregnancy rate was highly correlated with farrowing rate, an early indication of relatively low fertility in a small proportion of boars could be identified at an early stage of gestation.

Total litter size was different among boars and over time. Total litter size, rather than live born, was used as the measure of fertility on the assumption that the number of stillborn pigs is not likely determined by boar-dependent factors. This is particularly critical when gilts are used for the fertility evaluations, as problems at farrowing of these maiden females might contribute to substantial differences in numbers of stillborn pigs. The lower numbers born to the first breedings compared to subsequent breedings probably reflected the immaturity of the boars. The lower numbers born in the last replicate may relate to the cumulative effect of the high frequency of collection (twice-weekly) for relatively young animals. However, this program of collection provided adequate volumes of semen to rapidly identify the relatively less fertile boars.

The correlation between both pregnancy and farrowing rates, and total litter size born was not strong, as previously reported by Juonala et al. (1998), suggesting that for the more fertile boars these fertility characteristics may be differentially affected by semen quality. However, for boars G-1 and R-1 with the lowest relative fertility, all three measures of fertility were affected, suggesting that in less fertile boars a significantly lower pregnancy rate at Day 30 will likely be associated with a reduction in numbers of born.

Based on the fertility data alone, our results suggest that these less fertile boars could be identified with as few as twenty single-boar matings using relatively low sperm numbers for AI. Furthermore, using a standardized breed-abort protocol already established in our research group, cyclic gilts from the gilt development

program can be used to provide data on pregnancy rate and potential litter size when aborted at Day 30 of gestation. Thus, meaningful information on relative boar fertility can probably be obtained without waiting for the bred gilts to farrow, and this approach will have little impact on breeding herd productivity.

The results of routine laboratory evaluation in previous studies confirm that when sperm motility at collection is higher than 60 %, it is not predictive of boar fertility when 2 billion or higher sperm numbers per AI dose are used (Flowers, 1995; Flowers, 1997; Flowers and Tuner 1997; Xu et al., 1998; Popwell and Flowers, 2004). However, Tardif et al. (1999) reported that when 0.3 x 10^9 sperm per AI dose were used, the percent sperm with normal motility was positively correlated with farrowing rate (r=0.783, P=0.01). Similarly, although Berger and Parker (1989) and Popwell and Flowers (2004) found no correlation between morphology and fertility, Xu et al. (1998) demonstrated that the percentage of normal sperm was positively correlated with farrowing rate. In the present study, both the motility of raw semen and percentage of morphologically normal sperm were affected by a significant boar by time interaction: Therefore, in light of the very consistent differences in relative boar fertility *in vivo* over time, these laboratory assessments of semen quality would not be useful for predicting boar fertility.

In contrast to motility estimates with raw or extended semen on the day of collection, sperm motility in extended semen at Day 7 was correlated with *in vitro* fertility estimates (Xu et al., 1996b), but did (Juonala et al., 1998) or did not (Xu et al., 1998), correlate with *in vivo* fertility. Our results support the suggestion that sperm motility at Days 7 and 10 offers a practical approach for identifying relative boar fertility. However, more studies are needed to confirm this relationship and should involve precise methods of measuring sperm motility and motility characteristics, such as computer-assisted semen analysis (CASA).

Considering the IVF data, the *in vitro* characteristics that were not affected by time, but were different among boars, were potentially useful as predictors of fertility

in vivo. Male pronuclear formation rate is the only IVF variable that explains from 12 to 17% of the variation of fertility *in vivo* in both independent and multiple linear regressions evaluated. Overall, other IVF characteristics lacked strong correlations with *in vivo* fertility, suggesting that thresholds for sperm quality were being met when relatively fertile boars are compared. However, the lower fertility boars (G-1 and R-1) showed the lower values for oocyte penetration, MPN-f and >1MPN, and critical thresholds (e.g. >50 % oocyte penetration rate) can still possibly be used to identify subfertile boars.

The processes involved in IVF compared to *in vivo* fertilization may also contribute to the low correlations between *in vivo* and IVF data. Existing IVM and IVF systems have often been optimized for assessing oocyte quality and embryo production potential (Ding and Foxcroft, 1992; Ding et al., 1992; Rath, 1992; Ding et al., 1994ab; Abeydeera and Day, 1997; Zak et al., 1997; Long et al., 1999; Abeydeera, 2001), as in the present study, there is a need to improve IVM/IVF techniques for sperm evaluation. The use of standardized total sperm numbers per oocyte for IVF, without any adjustments for motility after sperm capacitation, *in vitro*, would probably help to identify the variation in sperm quality between the boars that affect the efficiency of fertilization process. Another approach could be to use much lower numbers of sperm per oocyte for IVF, thus placing the sperm in similar challenging situations *in vitro* and *in vivo*. Likewise, the use of the same ejaculate fractions for both *in vivo* and *in vitro* fertility evaluations could confirm the relationship between them. Recent studies by Rodríguez-Martínez et al. (2005) demonstrate that the sperm from the SP fraction present better laboratory characteristics (sperm membrane integrity, % of live cells, etc) than sperm from the bulk ejaculate (including subsequent Sperm-Rich fractions and Sperm-Free fractions of the ejaculate). These results could provide a better understanding of why this fraction presents the least variability when used for *in vitro* fertilization (Xu et al., 1996a), and represents the best sperm subpopulation to test in order to obtain a predictor of fertility. The difference between these fractions is likely produced by seminal plasma components (Zhu et al 2000; Rodríguez-Martínez et al., 2005). Therefore, further investigation

need to be done in this area to get a better understanding of the effects of sperm and seminal plasma interactions *in vivo*.

Certain limitations were encountered in evaluating zona pellucida penetration rates. Firstly, a limited area (approximately 30%) of each oocyte was evaluated; secondly, some of the sperm scored as penetrated could have only partially penetrated the zona and be misinterpreted as fully penetrated and finally, oocytes with a high number of sperm were difficult to score. All these technical limitations could be responsible for the lack of correlation between zona penetration rate and fertility *in vivo*. Likewise, this could explain the low correlation presented between zona penetration rate and fertility *in vitro* (oocyte penetration rate $r=54$, $P<0.0001$). The use of fluorescent stains to differentiate between the sperm binding to the zona and penetrated sperm could increase the accuracy of this test (Ivannova and Mollova, 1993), but unfortunately, this technique could be expensive and time consuming. Another option might be to evaluate the number of sperm binding to the zona pellucida. This evaluation has been found to be a useful indicator of *in vitro* fertility and embryo quality (Ardón et al., 2005; Braundmeier et al., 2005) and could be an alternative technique to be tested as a predictor of boar fertility.

In summary, we conclude that 1) The present study provides compelling evidence that appropriate changes to standard AI procedures, and specifically the use of low sperm numbers per dose for AI, will allow relatively subfertile boars to be effectively identified. 2) Objective methods for assessing progressive sperm motility in stored extended semen may be an effective indicator of relatively less fertile boars. 3) There is still a need to optimize existing IVF techniques for use as good predictors of boar fertility and semen quality. 4) Opportunities exist to develop timely and cost-effective procedures for excluding less fertile boars from commercial boar studs and further evaluation of these procedures is warranted.

Table 3-1. *In vivo* results from the nine boars during the evaluation period.

Boar	Number of Gilts			Fertility *in vivo*			
	Bred	Pregnant	Farrowed	Pregnancy rate (%)	Farrowing rate (%)	Total born (mean±SE)	Fertility index (mean±SE)
R-2	51	50	50	98 ^{x}	98 ^{x}	11.7±0.4 ^{ab}	11.4±0.6^{a}
Y-2	53	48	47	91 ^{xy}	89 ^{xy}	12.0±0.5 ^{a}	10.9±0.6^{ab}
Pu-3	57	54	52	95 ^{xy}	91 ^{xy}	11.2±0.5 ^{abc}	10.2±0.6^{ab}
B-1	55	54	52	98 ^{x}	94 ^{xy}	10.7±0.5 ^{abcd}	10.2±0.6^{ab}
R-3	55	52	52	94 ^{xy}	95 ^{xy}	10.9±0.4 ^{abcd}	10.1±0.6^{ab}
G-2	45	42	41	93 ^{xy}	91 ^{xy}	10.1±0.5 ^{abcd}	9.5±0.6^{ab}
B-3	55	51	51	93 ^{xy}	93 ^{xy}	9.6±0.5 ^{cd}	8.8±0.6^{abc}
R-1	56	48	47	86 ^{yz}	84 ^{yz}	10.0±0.4 ^{bcd}	8.4±0.6^{bc}
G-1	51	37	36	72 ^{z}	71 ^{z}	8.4±0.6 ^{d}	6.0±0.6^{c}
				P =0.0003	P =0.0003	P < 0.001	P <0.0001

$^{x-z}$: Means with different superscripts within each column were different by χ^2 analysis (P <0.05).
$^{a-b}$: LSM with different superscripts within each column were different (P <0.05). Values in the table are least square means (LSM) ± standard errors (SE) of LSM. P: probability of main effect of boar.

Table 3-2. Combined *in vivo* results from all nine boars for each breeding period (time) during the evaluation.

Time	Number of Gilts			Fertility *in vivo*			
	Bred	Pregnant	Farrowed	Pregnancy rate (%)	Farrowing rate (%)	Total born* (mean±SE)	Fertility index (mean±SE)
1	78	65	63	83	81	9.5±0.4	7.8±0.5 [a]
2	59	53	52	90	88	10.9±0.5	10.0±0.5 [ab]
3	65	58	58	89	89	10.6±0.4	9.5±0.5 [ab]
4	70	67	65	96	92	11.1±0.4	10.4±0.5 [b]
5	72	71	70	99	97	11.0±0.4	10.7±0.5 [b]
6	58	54	53	93	91	10.5±0.4	9.7±0.5 [ab]
7	76	68	67	89	88	9.7±0.4	8.7±0.5 [ab]
				P =0.13	P =0.14	P =0.04*	P =0.01

[a-b]: LSM with different superscripts within each column were different (P <0.05) using a pdiff adjusted by Tukey. *: Total born presented a significant main effect but no significant differences were found when LSM were compared using pdiff adjusted by Tukey. Values in the table are least square means (LSM) ± standard errors (SE) of LSM. P: probability of main effect of time.

Table 3-3. Semen characteristics of ejaculates collected from the nine boars during the evaluation period.

Boar	N	Total sperm per ejaculate (x 10^9)	Sperm with cytoplasmic droplets (%)	Motility Day 3 (%)	Motility Day 7 (%)	Motility Day 10 (%)
R-2	45	19 ± 1.5^{cd}	0.24 ± 0.12^{a}	79 ± 1.3^{a}	71 ± 1.9^{a}	60 ± 2.0^{a}
Y-2	45	27 ± 1.5^{ab}	0.17 ± 0.12^{a}	77 ± 1.3^{ab}	70 ± 1.9^{a}	61 ± 2.0^{a}
Pu-3	48	21 ± 1.6^{bcd}	0.17 ± 0.12^{a}	72 ± 1.3^{bc}	64 ± 1.9^{abc}	55 ± 2.1^{ab}
B-1	48	21 ± 1.6^{db}	0.27 ± 0.12^{a}	71 ± 1.3^{bc}	61 ± 1.9^{bc}	52 ± 2.3^{abc}
R-3	48	23 ± 1.6^{abc}	0.19 ± 0.12^{a}	76 ± 1.3^{ab}	67 ± 1.8^{ab}	60 ± 2.1^{a}
G-2	45	18 ± 1.5^{cd}	0.38 ± 0.12^{a}	76 ± 1.3^{ab}	67 ± 1.9^{ab}	55 ± 2.0^{ab}
B-3	48	29 ± 1.5^{a}	0.15 ± 0.12^{a}	73 ± 1.3^{abc}	66 ± 1.9^{ab}	59 ± 2.1^{a}
R-1	48	14 ± 1.5^{d}	0.35 ± 0.13^{a}	62 ± 1.5^{d}	55 ± 2.0^{c}	47 ± 2.5^{bc}
G-1	48	20 ± 1.5^{cd}	1.25 ± 0.12^{b}	69 ± 1.3^{c}	59 ± 1.9^{bc}	42 ± 2.3^{c}
		P <0.0001	P <0.0001	P <0.0001	P <0.0001	P <0.0001

N: number of ejaculates evaluated. P: probability of main effect of boar
^{abcd}: LSM with different superscripts within each column differ (P <0.05).
Values in the table are least square means (LSM) ± standard errors (SE) of LSM

Table 3-4. Boar by time effect (P =0.02) on percentage of morphologically normal sperm on the day of collection (d 0).

	Boars								
Time	R-2	Y-2	Pu-3	B-1	R-3	G-2	B-3	R-1	G-1
1	97 ± 1.1^{a}	99 ± 1.1^{a}	98 ± 1.5^{a}	95 ± 1.3^{a}	98 ± 1.5^{a}	96 ± 1.1^{a}	99 ± 1.3^{a}	87 ± 1.3^{b}	$86 \pm 1.3^{b\ y}$
2	99 ± 1.2^{a}	98 ± 1.2^{a}	99 ± 1.3^{a}	95 ± 1.3^{ab}	98 ± 1.3^{a}	99 ± 1.2^{a}	98 ± 1.3^{a}	89 ± 1.3^{b}	$90 \pm 1.3^{b\ xy}$
3	99 ± 1.3^{a}	99 ± 1.3^{a}	98 ± 1.1^{a}	96 ± 1.2^{ab}	99 ± 1.1^{a}	98 ± 1.3^{a}	98 ± 1.1^{a}	89 ± 1.2^{b}	$95 \pm 1.2^{ab\ x}$
4	98 ± 1.2^{a}	96 ± 1.2^{ab}	99 ± 1.2^{a}	97 ± 1.2^{a}	99 ± 1.1^{a}	98 ± 1.2^{a}	98 ± 1.1^{a}	88 ± 1.2^{b}	$93 \pm 1.2^{ab\ xy}$
5	99 ± 1.2^{a}	97 ± 1.2^{a}	98 ± 1.1^{a}	98 ± 1.2^{a}	98 ± 1.1^{a}	99 ± 1.2^{a}	99 ± 1.1^{a}	92 ± 1.2^{ab}	$87 \pm 1.2^{b\ y}$
6	99 ± 1.2^{a}	97 ± 1.2^{a}	97 ± 1.2^{ab}	99 ± 1.2^{a}	99 ± 1.2^{a}	98 ± 1.2^{ab}	99 ± 1.2^{a}	94 ± 1.3^{ab}	$92 \pm 1.2^{b\ xy}$
7	98 ± 1.3^{a}	99 ± 1.3^{a}	99 ± 1.3^{a}	98 ± 1.0^{a}	99 ± 1.3^{a}	97 ± 1.3^{a}	99 ± 1.3^{a}	90 ± 1.1^{b}	$94 \pm 1.0^{ab\ x}$

^{ab}: LSM±SE with different superscripts within each row were different (P <0.05).
$^{x-z}$: LSM±SE with different superscripts within each column were different (P <0.05).
Values in the table are least square means (LSM) ± standard errors (SE) of LSM.

Table 3-5. Effect of boar on IVF variables measured on at least 9 occasions during period of fertility assessment *in vivo*.

Boar	Zona Pellucida penetration (%)	Number of sperm penetrated the zona pellucida	Oocyte Penetration Rate (%)	Number of sperm penetrated per oocyte	Number of MPN per oocyte	Polyspermy Rate (%)	MPN-f Rate (%)	Potential embryo production (%)	1MPN (%)	>1MPN (%)
R-2	98±2.3^{abc}	8.8±4.7^{bc}	83±5^{b}	3.5±0.7^{b}	1.3±0.2^{b}	80±5^{b}	72±5.7^{ab}	14±4^{cd}	35.5±4^{bc}	37±5.9^{a}
Y-2	99±2.3^{a}	53.8±4.7^{a}	97±5^{a}	11.6±0.7^{a}	2.3±0.2^{a}	99±6^{a}	77±5.7^{a}	1±4^{d}	22.7±4^{c}	54±5.9^{a}
Pu-3	85±2.6^{d}	3.1±5.3^{c}	69±5^{bc}	1.6±0.8^{b}	1.1±0.2^{b}	40±6^{d}	79±6.4^{ab}	43±5^{ab}	54.8±4^{a}	24±6.7^{bc}
B-1	98±2.4^{abc}	26.5±5.0^{bc}	85±6^{ab}	3.1±0.8^{b}	0.9±0.2^{b}	74±7^{bc}	59±6.8^{bc}	15±5^{cd}	36.4±4^{abc}	22±7.1^{bc}
R-3	89±2.6^{cd}	11.0±5.3^{bc}	76±5^{bc}	2.1±0.8^{b}	1.3±0.2^{b}	46±6^{cd}	83±6.4^{a}	40±4^{ab}	54.8±4^{a}	26±6.7^{a}
G-2	96±2.3^{abc}	6.0±4.7^{c}	79±5^{bc}	2.6±0.7^{b}	1.3±0.2^{b}	61±5^{bcd}	76±5.7^{ab}	19±4^{cd}	32.7±4^{bc}	42±5.9^{ab}
B-3	87±2.6^{bcd}	6.4±5.3^{bc}	81±5^{b}	2.7±0.8^{b}	1.3±0.2^{b}	66±6^{bcd}	79±6.4^{ab}	24±4^{abc}	47.3±4^{ab}	32±6.7^{abc}
R-1	99±2.6^{ab}	29.2±5.3^{b}	75±5^{bc}	2.9±0.8^{b}	1.0±0.2^{b}	45±6^{cd}	56±6.8^{bc}	25±5^{abc}	36.8±4^{abc}	19±7.1^{bc}
G-1	97±2.4^{abc}	15.3±5.0^{bc}	49±6^{c}	1.8±0.8^{b}	0.6±0.2^{b}	36±6^{d}	48±6.8^{c}	24±5^{abc}	34.5±4^{abc}	11±7.1^{c}

MPN: male pronuclei per penetrated oocyte.
MPN-f: percentage of penetrated oocytes with at least one male pronucleus.
1MPN: percentage of penetrated oocytes with one male pronucleus.
>1MPN: percentage of penetrated oocytes with more than one male pronucleus.
$^{a-d}$: Means with different superscripts within each column were different ($P < 0.001$).
Values in the table are least square means (LSM) ± standard errors (SE) of LSM

Table 3-6. Multiple linear regression analyses of selected IVF variables (variables that presented differences among boars but not differences in time and no boar by time interactions) and fertility *in vivo* using a stepwise regression model.

Parameters	Pregnancy rate (%)	Farrowing rate (%)	Total born	Fertility Index (%)
		Correlation coefficient (r)		
Average SZP	/	/	/	/
Average SPO	/	/	/	/
Average MPN	0.405	0.410	0.306	/
MPN-f (%)	/	/	/	0.350
>1MPN (%)	0.16**	0.17**	/	/
Model R^2	0.16**	0.17**	0.09**	0.12**

Average SZP: average number of sperm penetrating the zona pellucida. Average SPO: average number of penetrated sperm per oocyte. Average MPN: average number of male pronuclei. MNP-f: percentage of male pronuclear formation. >1MPN: percentage of oocytes with more than one male pronucleus. Variables left in the model were significant at the 0.15 level. /: Variables that did not meet the 0.15 significance level for entry into the model. **:Model P value <0.005.

Table 3-7. Multiple linear regression analyses of selected sperm routine evaluation variables (variables that presented differences among boars, but not differences over time and no boar by time interactions) and fertility *in vivo* using a stepwise regression model.

Parameters	Pregnancy Rate (%)	Farrowing Rate (%)	Total Born (%)	Fertility Index
	Correlation coefficient (r)			
Cytoplasmic droplets (%)	/	-0.185	-0.219	-0.251
Motility at Day 3[a] (%)	/	/	/	/
Motility at Day 7[b] (%)	/	0.362	0.416	0.458
Motility at Day 10[c] (%)	0.346	/	/	/
Model R^2	0.12**	0.17**	0.22**	0.27**

[abc]: sperm motility (%) of extended semen on Days 3, 7, and 10, respectively. Variables left in the model were significant at the 0.15 level. /: Variables that did not meet the 0.15 significance level for entry into the model. **: Model P value <0.005.

Table 3-8. Multiple linear regression analyses of all selected sperm parameters (routine semen evaluation and IVF variables that presented differences among boars but not differences over time and no boar by time interactions) and fertility *in vivo* using a stepwise regression model.

Parameters	Pregnancy rate (%)	Farrowing Rate (%)	Total born (%)	Fertility Index
	Correlation coefficient (r)			
Average SZP	/	/	/	/
Average SPO	/	/	/	/
Average MPN	/	/	/	/
MPN-f (%)	0.405	0.410	/	0.187
>1MPN (%)	/	/	/	/
Cytoplasmic droplets (%)	/	-0.185	-0.253	-0.320
Motility at Day 3[a] (%)	/	/	/	/
Motility at Day 7[b] (%)	/	0.260	/	0.452
Motility at Day 10[c] (%)	0.254	/	0.421	/
Model R^2	0.23**	0.24**	0.24**	0.34**

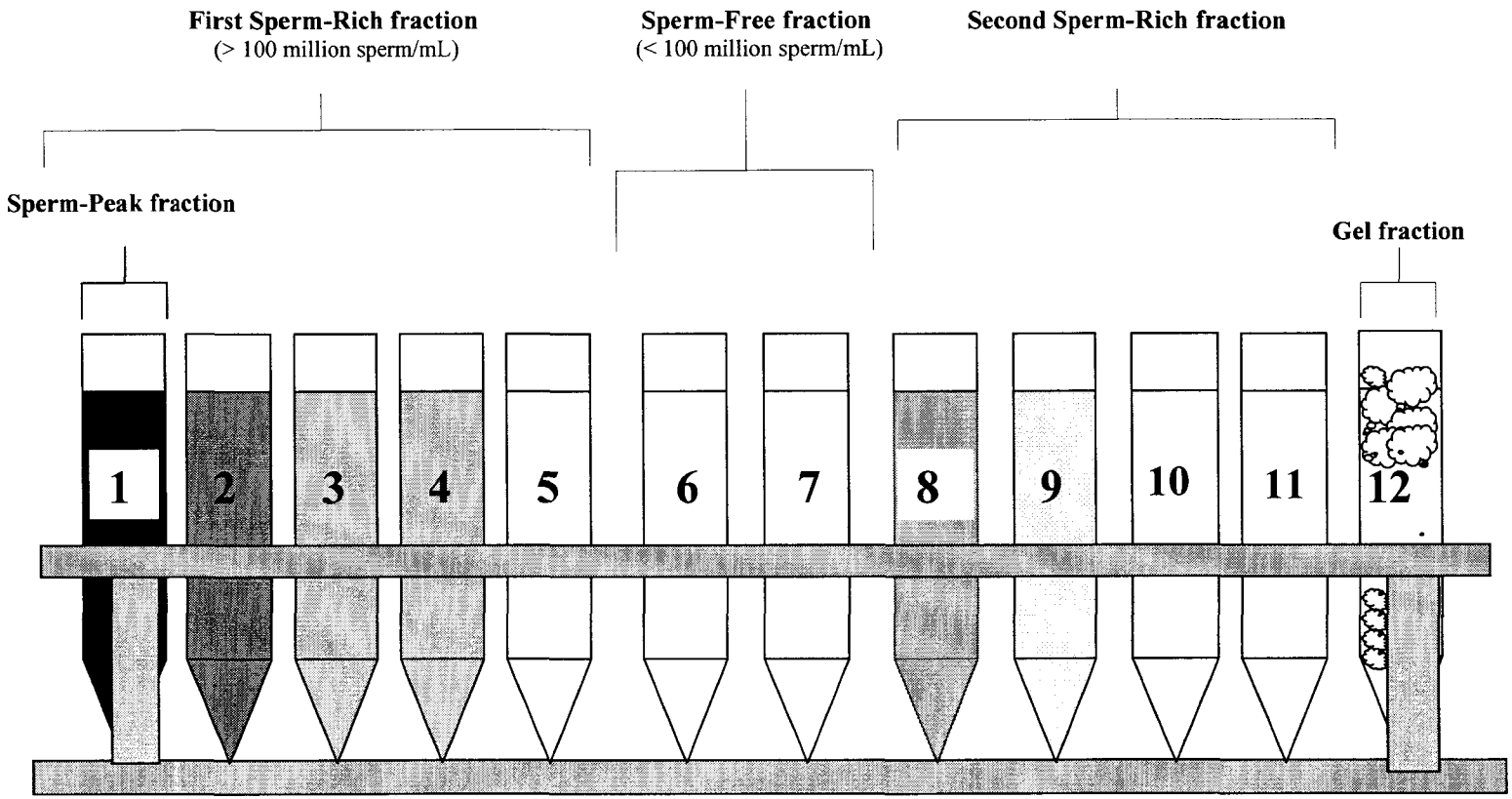
[abc]: sperm motility (%) of extended semen on Days 3, 7, and 10, respectively. Average SZP: average number of sperm penetrating the zona pellucida. Average SPO: average number of penetrated sperm per oocyte. Average MPN: average number of male pronuclei. MNP-f: male pronuclear formation rate. >1MPN: percentage of oocytes with more than one male pronucleus. Variables left in the model were significant at the 0.15 level. /: Variables that did not met the 0.15 significance level for entry into the model. **: Model P value <0.005.

Table 3-9. Independent linear regressions of selected semen variables (variables that presented differences among boars but not differences over time and no boar by time interactions) with fertility *in vivo* parameters.

Parameters	Pregnancy rate	Farrowing rate	Total born	Fertility Index
	Correlation coefficient (r)			
Cytoplasmic droplets (%)	-0.266*	-0.253*	-0.298*	-0.338*
Motility Day 3[a] (%)	0.314*	0.352*	0.277*	0.379*
Motility Day 7[b] (%)	0.326*	0.362*	0.416*	0.457*
Motility Day 10[c] (%)	0.343*	0.297*	0.378*	0.397*
Average SZP	0.032	0.043	0.228	0.159
Average SPO	0.052	0.069	0.282*	0.103
Average MPN	0.243	0.262*	0.306*	0.308*
MPN-f (%)	0.405*	0.410*	0.231	0.350*
>1MPN (%)	0.264*	0.280*	0.254*	0.295*

[abc]: sperm motility (%) of extended semen on Days 3, 7, and 10, respectively. Average MPN: average number of male pronuclei. MNP-f: male pronuclear formation rate. >1MPN: percentage of oocytes with more than one male pronucleus. Average SZP: average number of sperm penetrating the zona pellucida. Average SPO: average number of penetrated sperm per oocyte. *: $P < 0.05$.

Figure 3-1. Different fractions of an ejaculate used for analysis *in vivo* and *in vitro*. A complete single ejaculate was collected into tubes and different fractions were identified using a calibrated colorimeter. Fractions were identified as follows: The first sperm-rich fraction (SR), the sperm-peak (SP) fraction (tube containing the highest sperm concentration within the SR fraction), the sperm-free fraction (SF) (as the tubes after SR and before the second sperm rich fractions started that contain less than 100 x 10^6 spermatozoa per mL), and the gel fraction.



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CHAPTER FOUR

ANALYSIS OF SEMINAL PLASMA PROTEINS AS POTENTIAL MARKERS OF RELATIVE FERTILITY IN BOARS

4.1. INTRODUCTION

Reliable indicators of ejaculate quality allow exclusion of low quality ejaculates for use in AI (Kubus, 1993; Brooks, 1990; Mortimer, 1994; Flowers, 1995, 1997). However, effective predictors of semen quality and relative boar fertility are still needed (Ruiz-Sánchez et al., 2006). Seminal plasma is a complex fluid formed by the secretions of the male accessory sex glands and epididymis, which contains organic and inorganic components that have effects on sperm quality. Seminal plasma proteins are another important secreted component of seminal plasma and play an important role during sperm capacitation and fertilization (Rodríguez-Martínez et al., 1998). Specific proteins in seminal plasma have been identified as potential markers of male fertility in other species. Killian et al. (1993) identified two seminal plasma proteins (26 kDa, pI 6.2 and 55 kDa, pI 4.5) that were positively correlated with high fertility bulls and two proteins (16 kDa pI 4.1 and 16 kDa pI 6.7) that were correlated with low fertility. The 55kDa fertility-associated protein has since been identified as osteopontin (Cancel et al., 1999) and the 26kDa fertility-associated protein as Lipocalin-type prostaglandin D synthase (Gerena et al., 1998). In a study with stallions, Brandon et al. (1999) reported that SP-1, a 72 kDa, pI 5.6 seminal plasma protein, was positively correlated with fertility and demonstrated its antigenic homology with the bull 55 kDa fertility-associated protein. Furthermore, three other proteins (75 kDa, pI 6.0; 18 kDa, pI 4.3; 16 kDa, pI 6.5) were found to be negatively correlated with stallion fertility. In the boar, Flowers (2001) identified two boar seminal plasma proteins (55 kDa, pI 4.5 and 26 kDa, pI 6.2) that were positively associated with *in vitro* and *in vivo* fertility. Earlier, Flowers (2001) demonstrated that mixing seminal plasma of high fertility ("dominant") boars with the sperm of low

fertility ("non-dominant") boars improves the fertility of the low fertility boar sperm used for *in vivo* fertilization. Likewise, Zhu et al. (2000) found differences in oocyte penetration rates *in vitro* when sperm were pre-incubated with different fractions of seminal plasma from the same ejaculate. These studies confirm the direct effect of seminal plasma components on sperm quality, as determined by differences in fertility *in vitro.*

Specific proteins from the spermadhesin family, such as porcine seminal protein (PSP), AWN, and AQN, coat the sperm surface during ejaculation, producing structural changes to the sperm plasma membrane that affect sperm performance during the fertilization process (Maňásková et al., 2003). PSP-I, isolated and identified from boar seminal plasma by Rutherfurd et al. (1992), may prevent premature capacitation and the acrosome reaction (Kwok et al., 1993; Töpfer-Petersen et al., 1998). It may also have immuno-regulatory activity (Kwok et al., 1993; Yang et al., 1998; Assreuy et al., 2002, 2003). Multiple forms of PSP-I result from differences in carbohydrate moieties that could be related to these different functions (Rutherfurd et al., 1992; Nimtz et al., 1999). AWN-1 binds to β-galactosidases (Calvete et al., 1996a; Ekhlasi-Hundrieser et al., 2005) and to a O-linked oligosaccharides of the zona pellucida (Dostálová et al., 1995; Calvete et al., 1996b) and is the only spermadhesin present on the epididymal spermatozoa (Dostálová et al., 1995). This suggests possible zona-pellucida-binding activity (Sánz et al., 1992; Rodríguez-Martínez et al., 1998). AWN-1 may also have a role as a decapacitation factor (Töpfer-Petersen et al., 1998; Calvete et al., 1997). AWN-1 is synthesized by the rete testis, prostate, seminal vesicles (Sinowatz et al., 1995) and female reproductive tract (Ekhlasi-Hundrieser et al., 2002).

This information leads to the hypothesis that differences in relative boar fertility, or the quality of specific ejaculates or ejaculate fractions, could be related to differences in total seminal plasma proteins and/or specific fertility-related seminal plasma proteins. Furthermore, the varying effects of different ejaculate fractions on

sperm fertility could also be related to the total protein concentration and profiles in each fraction.

Therefore, the main objective of the present study was to evaluate seminal plasma proteins that could be effective predictors of relative boar fertility using: 1) a population of boars that would be considered acceptable for use in AI programs on the basis of ejaculate/sperm characteristics measured in most commercial AI centers, and 2) only 1.5 billion sperm per AI dose, to better reveal differences in relative boar fertility *in vivo* (Ruiz-Sánchez et al., 2006). As an extension of the earlier results of Zhu et al. (2000), seminal plasma proteins in three different fractions of the ejaculate were also compared.

4.2. MATERIALS AND METHODS

4.2.1. Boar fertility evaluation in vivo

The present study was an integral part of the comprehensive experiment described in Chapter 3 (Ruiz-Sánchez et al., 2006). Briefly, nine Genex Large White boars housed at the Swine Research and Technology Center (University of Alberta, Canada) were evaluated in three groups of three boars each (Blue-1, Red-1, Green-1; Red-2, Green-2, Yellow-2; Blue-3, Red-3 and Purple-3) during a 6.5 ± 0.1 mo period from May to February. A standardized AI protocol was used to breed 50 ± 5 gilts per boar during this time to determine fertility differences among boars. A number of ejaculate characteristics were also studied as potential markers of fertility *in vivo*. A total of 15 to 16 groups of gilts were bred during the evaluation period. To increase the number of gilts bred and farrowed per boar during specific breeding periods, that were subsequently used to correlate *in vivo* estimates of boar fertility to laboratory observations and analysis of seminal plasma proteins, data were analyzed from 4 time intervals (boar age); these represented successive periods from the start of breeding (time 1; 29-32 wk old) to the last evaluation period (time 4; 64-68 wk old).

4.2.2. Semen collection for seminal plasma evaluation

Ejaculates were collected into sterile pre-warmed 15-mL Falcon tubes (VWR Canlab, Mississauga, Ontario) as described by Xu et al. (1996ab) and three fractions were defined on the basis of sperm concentration as described previously in Chapter 3. Briefly, tubes containing the first Sperm-Rich fraction were identified both visually and using a calibrated colorimeter (model 254 Sherwood Scientific Ltd. Cambridge, UK). The Sperm-Peak fraction was considered to be the tube containing the highest sperm concentration within the Sperm-Rich fraction. The last tubes of the first Sperm-Rich fraction were measured to identify the final tube with a concentration of ≥100 x 10^6 spermatozoa per mL; this and the previous tubes were included as part of the first Sperm-Rich fraction. Finally, tubes containing less than 100 x 10^6 spermatozoa per mL, between the first Sperm-Rich fraction and the second Sperm-Rich fraction, were considered to be part of the Sperm-Free fraction as illustrated in Figure 4-1. All the tubes from the first Sperm-Rich fraction were then combined in a pre-warmed thermos, filtered through gauze to eliminate any gel component, and this combined first Sperm-Rich fraction was then used for routine semen evaluation (twice a week during the evaluation period), and for breeding by AI (every three weeks out of four).

On four occasions during the evaluation period (representing successive boar age from the start of breeding (time 1; 29-32 wk old) to the last evaluation period (time 4; 64-68 wk old), 3 mL aliquots of each identified fraction (Sperm-Peak; the combined Sperm-Rich, and the Sperm-Free) were used for seminal plasma protein evaluation. After collection, semen samples were centrifuged at 1,020 x g for 30 min at 4°C and the seminal plasma supernatant recovered was frozen with liquid nitrogen and stored at –20°C until analysis. Optimum centrifugation speeds for preparation of seminal plasma samples for analysis was determined by a pilot study, the results for which are presented in Appendix 3 Table A-3.

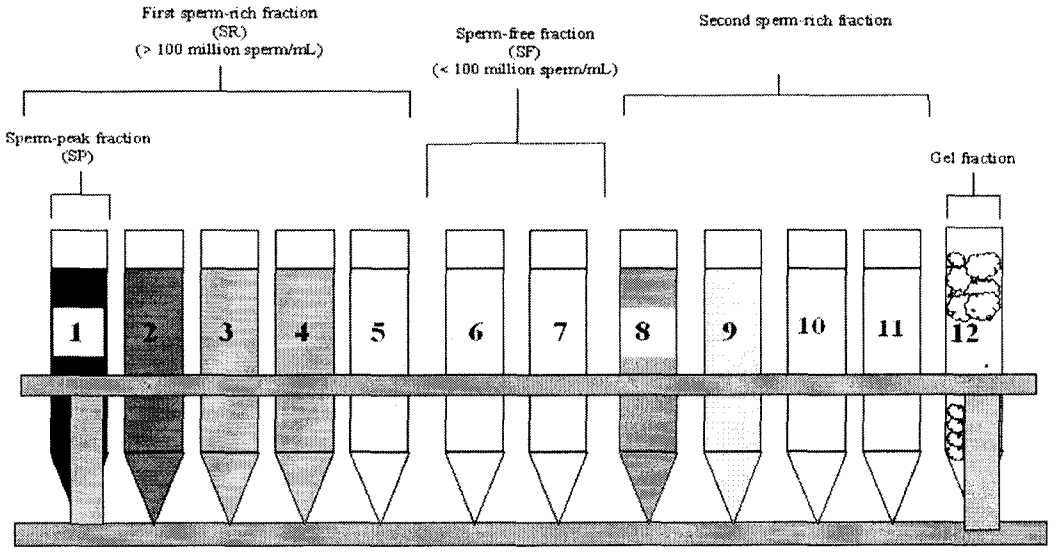


Figure 4-1. Different fractions of an ejaculate used for seminal plasma protein analysis and for *in vivo* and *in vitro* evaluation. A complete single ejaculate was collected into tubes and different fractions were identified using a calibrated colorimeter. Fractions were identified as follows: The first Sperm-Rich (SR) fraction, the Sperm-Peak (SP) fraction (tube containing the highest sperm concentration within the SR fraction), the Sperm-Free fraction (SF) (as the tubes after the SR fraction that contained less than 100 x 10^6 spermatozoa per mL), the second Sperm-Rich fraction, and the gel fraction.

4.2.3. Seminal plasma protein evaluation in vitro

4.2.3.1. Total protein evaluation

Seminal plasma samples were thawed and immediately centrifuged at 10,000 x g for 15 min to remove any cellular debris. Total protein concentration was then quantified using the Pierce BCA Protein Assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer's directions, using BSA as the standard. Sub-samples of seminal plasma containing 100 and 500 μg of total protein were then refrozen for subsequent analysis of specific seminal plasma proteins. See Table 4-1 for a graphic representation of seminal plasma fractions and boars evaluated for each seminal plasma protein analysis.

4.2.3.2. PSP-I analysis

4.2.3.2.1. Deglycosylation of PSP-I

Based on the preliminary studies of Zhu et al. (2000), an aliquot of each sample was deglycosylated to remove the N-linked oligosaccharides from the PSP-I glycoprotein, allowing quantification of a single PSP-I band by western blot analysis. Aliquots of 100 μg of total protein seminal plasma samples were diluted to a final concentration of 1 mg/mL with phosphate buffer (20 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.02 % (w/v) sodium azide) supplemented with 0.5% (w/v) SDS and 5% (v/v) β-mercaptoethanol and boiled for 2 min at 100°C to denature the proteins. Nonidet P-40 (final concentration of 2.5%) and 2 units of Peptide-N-Glycosidase (Roche Applied Science, Indianapolis, IN) were added and incubated for 16 to 18 h at 37°C. After treatment, the deglycosylated proteins were precipitated with three volumes of cold acetone for 45 min and centrifuged at 3,000 x g for 20 min. The pellet was washed twice with cold acetone and stored at –20°C until further analysis.

4.2.2.3.2. PSP-I Western blot analysis

The specific anti-PSP-I antibody used was kindly donated by Dr Kwok (Department of Obstetrics and Gynecology, Albert Einstein Medical Center, Philadelphia, USA). PSP-I was evaluated in both glycosylated and deglycosylated samples from the three fractions and four time periods (collections) from each of the nine boars. Both deglycosylated and glycosylated samples were separated on 15% (w/v) SDS-polyacrylamide gels. A total of 5 μg of total protein from each sample and a control (pooled) sample were loaded onto each gel. After electrophoresis, proteins were transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) using a constant current of 75mA for 15 h at 4°C. Membranes were blocked with Tris buffered saline (TBS) supplemented with 0.1% (v/v) Tween 20 (TBS-T) and 5% (w/v) non-fat milk for 1 h at room temperature. Membranes were then washed three times (5 min each) with TBS-T and incubated for 1 h with primary antiserum raised in rabbit against PSP-I (1:20,000 dilution), followed by three 5-min washes. The membranes were then incubated with anti-rabbit IgG,

peroxidase-linked donkey antibody (Amersham Pharmacia Biotech) for 30 min at room temperature (1:16,000 dilution), and washed three times with TBS-T for 5 min. Immunoreactivity was detected by chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The membrane was exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech) for 10 sec and developed. The films were scanned using an Imaging Densitometer (Bio-Rad Labs, Hercules, CA) and immunoactive PSP-I bands were quantified using densitometry analysis software (Molecular analyst v2.01, Bio-Rad Labs). To standardize the gels, an internal control (pooled) sample was run in each blot to provide a reference gel. The relative abundance of PSP-I (arbitrary units per μg of total protein) was calculated using the optical density of the sample divided by optical density of the internal control, divided by the amount of protein loaded (5 μg). The PSP-I concentration per mL of seminal plasma (PSP-I/mL) was estimated as PSP-I abundance x the total protein concentration. Finally, the total amount of PSP-I per AI dose (PSP-I/AI dose) was calculated as PSP-I/mL x estimated volume of seminal plasma included in each AI dose of semen. Seminal plama concentrations of AWN-1 and osteopontin were calculated in the same manner.

4.2.2.4. AWN-1 analysis

Due to limitations in the amount of AWN-1 antibody available (kindly provided by Dr F Sinowatz (Department of Veterinary Anatomy, University of Munich, Veterinarstrasse, Germany) and Dr E Töpfer-Petersen (Institut für Reproduktionsmedizin, Tierärzttliche Hochschule, Hanover, Germany), AWN-1 analysis was limited to seminal plasma samples of the two highest (R-2 and Y-2) and the two lowest (G-1 and R-1) fertility boars, based on their Fertility Index assessed *in vivo* (see Table 4-2); for each boar, three different seminal plasma fractions from four ejaculates collected were analyzed (Table 4-1). The Western blot protocol was as used for PSP-I with the following differences: The dilutions used for the first (AWN-1) and second antibody (rabbit anti-chicken IgG, IgY peroxidase antibody; A9046 Sigma Chemical, St Louis, Mo. USA) were 1:1,000 and 1:10,000, respectively.

4.2.2.5. Osteopontin analysis

Equivalent samples from the same four boars analyzed for AWN-1 were also evaluated for osteopontin (OPN) (Table 4-1), again using the PSP-I Western blot protocol with the following variations based on Johnson et al. (1999). Proteins (25 μg) were loaded onto each well and separated on a 15% (w/v) polyacrylamide gel. A sample of sow's milk was used as an OPN positive control. The membranes were initially blocked overnight at 4°C with TBS-T 5% (w/v) non-fat milk, incubated overnight with a cocktail of rabbit polyclonal antibodies against recombinant human OPN (LF-123 and LF-124 generously donated by Dr. LW Fisher (National Institute of Health, Bethesda, MD)) (1:1,000) and then incubated for 2 h at room temperature with a peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:10,000). The membrane was exposed to a Hyperfilm ECL for 15 sec. Specificity of the 1st antibody was previously tested by Cancel et al. (1997) to identify OPN in bull semen and by Johnson et al. (1999) to identify OPN in ovine uterus.

4.2.2.6. 2-D gel electrophoresis of seminal plasma proteins

Proteomic analysis of seminal plasma proteins using 2-D gel electrophoresis was used as a complementary test to provide important preliminary data on the abundance of specific proteins, without having to rely on existing antibodies for detection. 2-D gel analysis was restricted to the Sperm-Rich fraction of the same samples used for AWN-1 and OPN analysis.

The Sperm-Rich fraction was extracted using the same acetone precipitation method described earlier and 225 μg of protein for the 24 cm gels and 100 μg of protein for the 7 cm gels, were solubilized in rehydration buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 0.5 % (v/v) Pharmalytes pH 3-10, and 2.8 mg/mL dithiothreitol) for 1 h at room temperature, before loading the sample onto 7 or 24 cm linear pH 3-10 Immobiline (Amersham Biosciences) strips for overnight rehydration. The first dimension separation program on an Ettan IPGPhor isoelectric focusing apparatus (Amersham Biosciences) for the 7 cm strips was 10 min at 500 V, 10 min at 1,000 V, 1.5 h at 4000 V and 1 h at 5,000 V for a total of 11, 250 Vhr. The program

for the 24 cm strips included 100 V for 1 h, 250 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, 2,000 V for 1 h, 4,000 V for 2 h and 8,000 V for a total of 60,000 Vhr to reach a total focusing time of 68,000 Vhr. After focusing, strips were equilibrated in 50 mM Tris-HCl, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and bromophenol blue, for 15 min with 1% (w/v) DTT, and then alkylated using 2.5 % (w/v) iodoacetamide for 15 min. The gel strips were then loaded onto 12% (w/v) SDS-PAGE gels to separate proteins in the second dimension according to their molecular weight. The 7 cm strips were all run simultaneously using a 6-gel electrophoresis system, with three 7 cm strips run on each of six 24 cm slab gels in the second dimension. These slab gels were cast simultaneously using a 6-gel multi-caster (EttanDalt system, Amersham Biosciences). The 24 cm strips from the first dimension were run in three different replicates of six 24 cm slab gels, in order to accommodate all 16 samples. The resulting gels were then fixed overnight and proteins visualized using silver staining.

For each gel, the protein spots were selected and quantified using Imagemaster 2-D Elite analysis software (Amersham Biosciences). The 24 cm gels were compared to the 7 cm gels used in the first dimension to determine the quality of protein identification and separation. The final analysis was completed with data from the 7 cm gels, as the protein separation and visualization of the spots were superior. The gels were first examined using "averaged gels" which included protein spots that were present in at least 3 of the 4-gel series run for each boar over time. This analysis was used to determine the differences across all the protein species visualized between the highest and the lowest fertility boars at all four time periods. To analyze the differences in quantity of specific proteins across boar and treatment, specific protein abundance was quantified on all individual gels. The individual volume measurements (in relative units) for each protein on different gels were normalized using the same reference gel.

4.2.3. Statistical analysis

As mentioned in section 4.2.1 a total of 15 to 16 groups of gilts were bred during the evaluation period. To increase the number of gilts bred and farrowed per

boar during specific breeding periods that were subsequently used to correlate *in vivo* estimates of boar fertility to laboratory and *in vitro* assessment of semen quality, data were analyzed at 4 time intervals (boar age), representing successive periods from the start of breeding (time 1; 29-32 wk old) to the last evaluation period (time 4; 64-68 wk old). The results of *in vitro* fertilization and routine semen evaluation were grouped on the same basis for the statistical analysis.

Additionally, based on the Fertility Index developed for the same boars in Chapter 3, a sub-set of two "High" and two "Low" fertility boars was identified (fertility class) and used for further comparisons of seminal plasma proteins.

The differences of seminal plasma fractions (n=3) were analyzed using a repeated measures analysis by a mixed procedure of the Statistical Analysis System (SAS version 8.1, SAS Institute Inc. Cary, NC, USA). The fixed effects were time (4 levels), fractions (n=3), and their interaction; boar nested within fraction was used as the subject, and the boar group as a random effect. The influence of boars on total protein and PSP-I within fraction was analyzed as a repeated measures analysis using a mixed procedure of SAS. The fixed effects were time (4 levels), boar (n=9) and the boar group as a random effect. AWN-1, OPN and identify proteins on 2-D gel data were also analysed with repect to fertility class (High and Low fertility boars) and evaluated using a mixed procedure of SAS. The fixed effects were time (4 levels), fertility class (n=2), and their interaction; boar was used as the subject, and the boar group as a random effect. In all statistical models, the Kenward-Roger option was used to calculate the denominator degrees of freedom. The variance-covariance matrix was chosen for each statistical model by an interactive process wherein the best fitting model was based on Schwarz's Bayesian criteria. Least square means and standard errors were generated and separated using a pdiff adjusted by Tukey option for significant, fixed effects. All data are presented as LSmeans ± standard errors of LSmeans.

In vivo fertility for the subsets of of High and Low fertility boars was averaged

within boar (n=4) and time (boar age) (n=4) and the Insight procedure was used to evaluate the correlation between fertility in vivo (conception rate, farrowing rate, total pigs born, and Fertility Index) and seminal plasma proteins (Total protein, PSP-I, AWN-1, osteopontin and proteins identified in the 2-D gel system).

4.3. RESULTS

4.3.1. Fertility evaluation in vivo

For ease of interpretation of the present data, the relative fertility of the boars is shown in Table 4.2 and ranked according the Fertility Index described previously in Chapter 3 (Ruiz-Sánchez et al., 2006).

4.3.2. Seminal plasma protein evaluation

4.3.2.1. Western blot analysis

The specific antiserum against PSP-I showed immunoactivity against both glycosylated (PSP-I) and deglycosylated PSP-I proteins (D-PSP-I) in the range of 14 to 20 kDa and 12 kDa respectively (Figure 4-2).

The antiserum raised against AWN-1 showed immunoactivity against a specific band at 14 kDa (Figure 4-3).

Finally, the use of the polyclonal antiserum raised against the N-terminus (LF-124) and C-terminus (L-123) of human recombinant OPN showed immunoactivity against three protein bands of 70, 12 and 9 kDa (OPN-70, OPN-12 and OPN-9, respectively) (Figure 4-4).

4.3.2.2. Seminal plasma evaluation of all nine boars: Total protein, PSP-I and D-PSP-I

The seminal plasma fractions affected the concentrations of seminal plasma

proteins (P<0.0001). Concentrations of total protein, PSP-I and D-PSP-I were all lower (P<0.05) in the Sperm-Peak, than in the Sperm-Rich and Sperm-Free fractions (Table 4-3). The period of study (boar age) also affected the concentrations of total protein (P=0.01) (29, 32, 22 and 26 mg/mL, respectively, for time periods 1 to 4; pooled SEM =3). D-PSP-I abundance showed a fraction by time interaction (P<0.05; data not shown). Evaluation of effect of boar and time within fraction indicated that total protein and D-PSP-I concentration were not different (P>0.10) among boars in the Sperm-Peak and Sperm-Free fractions. However, there were differences in total protein concentration (P=0.025) and D-PSP-I concentration (P=0.014) among boars in the Sperm-Rich fraction (Table 4-2). No differences among boars were found in the relative abundance of D-PSP-I, or in the total amount of protein and D-PSP-I per AI dose for any fraction. Differences in time (P<0.05) were only found for D-PSP-I relative abundance for the Sperm-Peak and Sperm-Rich fractions.

D-PSP-I relative abundance in the Sperm-Rich fraction was negatively correlated with pregnancy rate (r=-0.46, P=0.005), farrowing rate (r=-0.43, P=0.007) and Fertility Index (r=-0.42, P=009). Sperm concentration and total protein concentration were negatively correlated when data of the Sperm-Peak and Sperm-Rich fractions were included in the model (r=-0.45; P=0.0001), confirming an inverse relationship between sperm concentration and the amount of protein in seminal plasma.

Results from Chapter 3 were used for the comparison of *in vitro* fertilization (IVF) data with seminal plasma protein data. This comparison established that total protein concentration was negatively correlated with zona pellucida penetration rate and average number of sperm penetrating the zona (r=-0.47 and r=-0.50, respectively; P<0.05). There was also a negative relationship between D-PSP-I abundance and average number of sperm penetrating the zona (r=-0.38, P<0.05).

4.3.2.3. Seminal plasma evaluation of the two highest fertility and two lowest fertility boars: AWN-1 and Osteopontin

Comparisons of seminal plasma fractions demonstrated that OPN-9 and OPN-12 concentrations were lower ($P<0.05$) in the Sperm-Peak fraction than in the other two fractions (Table 4-4). Concentrations of AWN-1 and OPN-70 were lower ($P<0.05$) in the Sperm-Peak fraction than the Sperm-Free fraction, whilst concentrations in the Sperm-Rich fraction were intermediate and not different from the other two fractions (Table 4-4). No difference in any measure of protein content among the highest and the lowest fertility boars in the Sperm-Free and Sperm-Peak fractions were established (Table 4-5), whereas differences were found for the Sperm-Rich fraction in AWN-1 concentration, and the lowest fertility boars had the highest amount of AWN-1, OPN-9 and OPN-12 per AI dose.

Relative abundance of OPN-70 in the Sperm-Peak fraction was negatively correlated with total born ($r=-0.47$, $P=0.02$) and Fertility Index ($r=-0.47$, $P=0.03$). However, in the Sperm-Rich and Sperm-Free fractions, AWN-1 and OPN relative abundance did not show any correlations with *in vivo* fertility. The estimated content of AWN-1, OPN-9 and OPN-12 per AI dose was negatively correlated with total born ($r=-0.45$, $r=-0.42$, $r=-0.43$, respectively; $P<0.05$) and Fertility Index ($r=-0.47$, $r=-0.49$, $r=-0.49$, respectively; $P<0.05$), and OPN-9 and OPN-12 were both negatively correlated with farrowing rate ($r=-0.46$, $r=-0.44$, respectively; $P<0.05$).

4.3.2. 2-D gel electrophoresis of seminal plasma proteins

Figure 4-5A is a representative 7 cm gel showing two-dimensional separation of seminal plasma proteins for the second collection from Boar R-1, with the numeric annotation of proteins corresponding to the numeric assignation of proteins presented in Table 4-6. From a qualitative perspective, all seminal plasma protein species were identified in both the highest and the lowest fertility boars, using the "pooled reference gels" described earlier. Of the 42 identified proteins quantified, there were differences ($P<0.05$) among boars for Protein 7 (46 kDa, pI 6.9), 17 (10 kda, pI 9.0), 22 (18 kDa, pI 9.2), and 24 (27 kDa, pI 7.6). One of the lowest fertility boars, R-1, had lower

abundance of seminal plasma Proteins 7, 17 and 22 ($P<0.05$) than the other three boars (Table 4-6). There were differences over time for Proteins 35, 36 and 40 ($P<0.05$), but no differences in relative abundance ($P>0.05$) of these proteins when analyzed by fertility class.

Western blot analysis performed on a duplicate of the representative 2-D gel showed in Figure 4-5A, produced immunoreactivity against PSP-I for a group of protein species that appeared to include Proteins 22 and 17 (Figure 4-5B). However, further verification of the identity of these proteins is required. As well, immunoreactivity against AWN-1 (Figure 4-5C) was detected in a duplicate of the same representative 2-D gel presented in Figure 4-5A.

When correlations with *in vivo* characteristics were considered, Protein 4 (60 kDa, pI 6.5) was negatively correlated with farrowing rate ($r=-0.66$, $P=0.04$) and with the Fertility Index ($r=-0.66$, $P=0.04$), while Protein 27 (22 kDa, pI 6.0) had a strong negative relationship with total born ($r=-0.77$, $P=0.010$). In contrast, Protein 26 (26 kDa, pI 5.9) tended to be positively correlated with pregnancy rate ($r=0.454$, $P=0.09$), farrowing rate ($r=0.450$, $P=0.09$) and Fertility Index ($r=0.481$, $P=0.07$).

4.4. DISCUSSION

As hypothesized, specific proteins in seminal plasma were associated with boar fertility *in vivo* and *in vitro*, providing the basis to use them as a complementary tool to identify sires with high and low relative fertility that would have considerable economic impact in the productivity of the farm.

As a part of the fertilization process, seminal plasma proteins play an important role during sperm reservoir formation, sperm capacitation, and sperm-oocyte interactions. Specific seminal plasma proteins have been identified as potential markers of male fertility in the bull (Killian et al., 1993), stallion (Brandon et al.,

1999) and the boar (Flowers, 2001). In the context of the data reported from the present study, it is important to emphasize two key points. Firstly, compared with the wider range of boar fertility and ejaculate quality used in previous studies (Flowers, 2001), the quality of the ejaculates used in the present study (>80% progressive motility and >85% morphologically normal sperm) exceeded normal industry standards for use in AI. Secondly, boar fertility was evaluated *in vivo* using a low sperm dose (1.5 billion morphologically normal, and motile, sperm per AI dose). It is essential to recognize that these specific conditions may identify a different subpopulation of relatively less fertile boars within the industry's normal standards for use in AI.

4.4.1. Profile of seminal plasma proteins differs among ejaculate fractions

In the present study, dividing the ejaculates in discrete fractions at collection allowed further characterization of differences in seminal plasma proteins among these fractions as previously reported by our laboratory (Zhu et al., 2000). The Sperm-Peak fraction contained lower total protein than the other two fractions and lower concentrations of all specific proteins than the Sperm-Free fraction. One explanation for these differences could be the origin of the seminal plasma components with respect to the different accessory sex glands and their sequential contribution of secreted proteins to the ejaculate. As reviewed by Setchell et al. (1988) and Shivaji et al. (1990) the first secretions present in the ejaculate are produced by the prostate gland in order to clean the male urino-genital tract, followed by very high sperm concentrations in the first 10 to 15 mL of the Sperm-Rich fraction. The ejaculate then becomes increasingly diluted by seminal vesicle fluid in the next 30 to 70 mL of the ejaculate, until essentially sperm free fluid is collected in the Sperm-Free fraction. Depending on the frequency of collection, a second, less concentrated Sperm–Rich fraction may be present, after which the ejaculate ends with a gel fraction being secreted from the bulborourethral glands. Prostate secretions contain lower concentrations of PSP-I and AWN-1 than seminal plasma of the complete ejaculate (Maňásková et al., 2002). These results support our observations that the Sperm-Peak fraction, the first sperm fraction collected that contains mainly prostate secretions, had

lower concentrations of PSP-I than the Sperm-Rich fraction. A second factor that could contribute to the protein differences between fractions is the variability in the concentration of sperm. The high concentration of sperm in the Sperm-Peak fraction would adsorb a higher proportion of proteins onto their membranes, thus reducing the residual amount of total protein in seminal plasma. Metz et al. (1990) reported that epididymal sperm adsorbed 14 pg protein/sperm over a 10 min period and that 82% of the proteins adsorbed were low molecular weight proteins. These authors also confirmed that low quality sperm (progressive motility of 50 to 30% after washing with Tyrode's solution) adsorbed significantly less protein (3 pg protein/sperm per 10 min). This relationship is consistent with our observations that sperm concentration and total protein concentrations were negatively correlated in the Sperm-Rich and Sperm-Peak fractions. Therefore, a combination of three factors: 1) seminal plasma origin, 2) sperm quality (sperm ability to absorb seminal plasma proteins), and 3) sperm concentration, collectively determine the variation in total protein concentration among the seminal plasma fractions.

4.4.2. Spermadhesin: PSP-I and AWN-1 abundance

PSP-I and AWN-1 are members of the spermadhesin protein family isolated from seminal plasma and may play an important role during fertilization (Töpfer-Petersen et al., 1998; Yang et al., 1998; Maňásková et al., 2000; Assreuy et al., 2002). PSP-I has multiple forms due to differences in its carbohydrate moiety (Rutherfurd et al., 1992; Nimtz et al., 1999). In the present experiment, glycosylated PSP-I species ranging from 20 kDa to 14 kDa, were identified. In contrast, by applying more stringent techniques than used previously (Zhu et al., 2000), a single deglycosylated form of PSP-I was present as a compact 12 kDa band, which allowed more accurate quantification of this protein (Figure 4-2). Western blotting results revealed that PSP-I and AWN-1 were detected in all seminal plasma fractions analyzed and the Sperm-Peak fraction contained the lowest concentration of these two proteins. These results reflect the observed differences in total protein concentration and may again be determined by the combination of the three factors discussed above; 1) seminal plasma

origin, 2) sperm quality (sperm ability to absorb seminal plasma proteins), and 3) sperm concentration.

4.4.3. Osteopontin

Osteopontin (OPN) has been detected in female (Johnson et al., 1999; Garlow et al., 2002) and male (Cancel et al., 1999; Rodríguez et al., 2000; Luedtke et al., 2002) reproductive tracts. OPN has been identified (Brandon et al., 1999) as a seminal plasma protein that is positively associated with fertility in the bull (55 kDa, pI 4.5) (Cancel et al., 1997; Cancel et al., 1999) and the stallion (SP-1, 72 kDa, pI 5.6). In the present experiment, western blot analysis with an antiserum against human OPN, identified three immunoactive bands at 70, 12 and 9 kDa. A 70 kDa form of OPN has been reported in uterine flushings in the pig (Garlow et al., 2002), and appears to be homologous to SP-1, a stallion seminal plasma osteopontin (Brandon et al., 1999). Although the 70 kDa osteopontin species identified in the present study of boar seminal plasma is homologous to the bull (55 kDa) and stallion (70 kDa) OPN form, the relative amount of OPN-70 did not differ among boars. This may reflect the fact that all the ejaculates used in the present study exceeded normal industry standards for use in AI (>80% progressive motility and >85% morphologically normal sperm) and also that the differences in fertility *in vivo* were established using low numbers of sperm per AI dose. As there were no major differences in the relative abundance of AWN-1, OPN-12 and OPN-9 in the ejaculates collected, differences in the concentration of these specific proteins in seminal plasma from the highest and lowest fertility boars, may largely reflect differences in total protein concentration, and the sperm concentration of each ejaculate, which ultimately determines the amount of seminal plasma present after dilution of the ejaculate to 1.5 billion sperm per 50mL AI dose.

4.4.4. Seminal plasma protein 2-D gel analysis and its relationship with fertility parameters

The analysis of seminal plasma proteins on 2-D gels provides important preliminary data on the abundance of specific proteins, without having to rely on

existing antibodies for detection. This approach was used successfully to identify proteins that were associated with fertility in the stallion (Brandon et al., 1999), and the bull (Killian et al., 1993). In contrast, analysis of 42 different proteins in the seminal plasma of two lowest and the two highest fertility boars in the present experiment failed to establish differences in the seminal plasma proteome that showed clear associations with established differences in boar fertility. Although four proteins were identified as differing among boars, none of these proteins were specifically related to fertility. Two of the proteins, Protein 17 and Protein 22 are in the same molecular size range and pI as PSP-I, and preliminary immunoblots confirmed the presence of PSP-I in this area of the gel (see Figure 4-6). Further studies are needed to confirm the identity and possible function of these proteins.

Based on immunoactivity to an antibody raised against recombinant human osteopontin (Figure 4-4), the 70 kDa protein identified appears to be the boar equivalent of osteopontin, reported as an effective marker of fertility in the stallion and bull. However, under the conditions used, no proteins in this range could be identified on the 2-D gels, and none of the other higher molecular weight proteins analyzed were associated with differences in fertility. Therefore, verification of this protein's identity will need to be pursued in a subsequent study. Flowers (2001) reported that differences in a 55 kDa, pI 4.5 protein appeared to be associated with differences in fertility *in vivo*, and this protein appears to be equivalent to Protein 35 identified by 2-D gel analysis in our study. The lack of an association with fertility in our study is likely due to the use of boars with a narrower range of fertility, thus excluding the more infertile boars that appeared to create most of the variance in both fertility and the amount of the 55 kDa protein in the experimental paradigm reported earlier by Flowers (2001).

Protein 26 (25 kDa, pI 5.9) identified in the present study tended to be positively associated with fertility. This protein appears to show homology with a protein that was positively associated with fertility in the boar (Flowers, 2001) and bull (Killian et al., 1993), and has since been identified in the bull as lipocalin-type

prostaglandin D synthase (Gerena et al., 1998). Although no role for this protein is yet confirmed in seminal plasma, the authors suggest that as it was immunolocalized on the apical ridge of the acrosome in bull sperm, it may play a role in both the development and maturation of spermatozoa (Gerena et al., 2000). Further studies are needed to verify the identity of Protein 26 observed in boar seminal plasma and to determine its possible use as a marker of sperm quality and boar fertility.

Interestingly, Protein 27 (20 kDa, pI 6.0), identified by 2-D gel analysis in the present study, showed a strong negative correlation (r=-0.7627, P=0.01) to total litter size born, but not to other fertility traits included in the overall Fertility Index. In contrast, the relative abundance of Protein 4 (60 kDa, pI 6.5) was negatively correlated with both farrowing rate and the overall Fertility Index. The identity of these two proteins also needs to be established, as they are also potential markers of relative fertility of boars that cannot presently be identified using traditional laboratory measures of ejaculate quality.

4.4.5. Total protein evaluation and PSP-I

Specific proteins have been described as decapacitation factors in the seminal plasma of humans (antifertility factor 1AF1), bulls (bull seminal plasmin (SPLN) and boars (decapacitation factor) (Bonilla et al., 1996), and are assumed to protect sperm from factors in the female tract that could trigger early capacitation, thus reducing the possibility of sperm-oocyte binding. These suggestions are consistent with the observations that pre-incubation of sperm from the Sperm-Peak fraction with seminal plasma from the Sperm-Free fraction reduced oocyte penetration rate *in vitro* (Zhu et al., 2000), whereas inclusion of seminal plasma to sperm samples sorted by flow-cytometry increased the percentage of uncapacitated, acrosome-intact sperm and reduced oocyte penetration rate in vitro (Maxwell and Johnson 1999). In the present experiment, total protein concentration of seminal plasma was negatively correlated with both zona pellucida penetration rate and number of sperm penetrating the zona *in vitro*, suggesting that an increase in total proteins produces a predominantly decapacitating effect. As a consequence, the increase in uncapacitated sperm could

decrease fertilization rate *in vitro*, albeit in a presumably transient manner. No differences were reported in sperm viability and mitochondrial activity when PSP-I was incubated with a highly diluted sperm sample (García et al., 2003). However, reports of a negative correlation between the number of sperm attaching to each oocyte, and PSP-I in seminal plasma (Zhu 2000), is consistent with the observation in the present study that D-PSP-I abundance was negatively correlated with both *in vivo* and *in vitro* fertility. Direct evaluation of sperm capacitation state, and the populations of specific seminal plasma proteins on the sperm membrane before and after capacitation, is needed to better understand the specific functions of seminal plasma proteins during the fertilization process. In terms of the overall differences in fertility among the boars used in the present study, the lack of differences in D-PSP-I abundance in the ejaculates, suggests that this protein is not a useful predictor of boar fertility in the experimental paradigm used.

4.4.6. Conclusion and practical perspectives

The overall results of the integrated study presented here and in Chapter 3, provide the first evidence for substantial differences in boar fertility that cannot be identified by existing laboratory techniques used in commercial boar studs. However, inadvertent use of the relatively sub-fertile boars identified in this study would substantially affect breeding herd performance. The proteomic analysis reported provides some of the first evidence of specific boar seminal plasma proteins that may mediate observed differences in semen quality. Although there have been preliminary reports that protein markers can identify markedly less fertile boars (Flowers, 2001), the ejaculate quality of the boars in the present experiment at collection tended to exceed the minimal accepted limits for AI use, as determined by routinely used laboratory criteria. The combined results from this study confirm that there is no single test that can apparently predict sperm quality or boar fertility among groups of relatively fertile boars. Fertilization in mammals is a complex process, involving multiple interactions between the sperm and the seminal plasma components of the ejaculate. Furthermore, extensive interactions between components of the ejaculate and the female reproductive tract *in vivo* are very different to the conditions in which

sperm maturation is carried out *in vitro*. Therefore, semen characteristics that favour successful IVF may not necessarily favour optimal fertility when the same sperm are used for AI. Also, from a practical perspective, if the trend towards using lower sperm numbers for intra-uterine insemination continues, it will be essential to identify boars that are markedly less fertile in this situation. This will allow the industry to capture the full benefit of using fewer, but higher indexed, boars. Information on the balance of proteins that determine the rate of capacitation and those that ultimately allow effective sperm binding to the zona pellucida and to the oocyte, will undoubtedly help to improve the selection of such high impact boars. The results presented in this Chapter identify several seminal plasma proteins that merit further investigation as markers of ejaculate quality and boar fertility.

Table 4-1. Seminal plasma fractions and boars evaluated for each protein. A total of four seminal plasma samples per boar were evaluated during the 6.5 ± 1 mo *in vivo* evaluation period.

Protein evaluation	Ejaculate fraction			Boars	
	Sperm-Peak	Sperm-Rich	Sperm-Free	9 boars	High and Low fertility
Total protein (n=108)	X	X	X	X	
PSP-I (n=108)	X	X	X	X	
AWN-1 (n=48)	X	X	X		X
Osteopontin (n=48)	X	X	X		X
2-D gel electrophoresis (n=8)		X			X

Table 4- 2. Main effects of total seminal plasma proteins and deglycosylated seminal plasma PSP-I (D-PSP-I) from the Sperm-Rich fraction of ejaculates collected during the evaluation period on relative boar fertility *in vivo*. The differences in Fertility Index for each boar are presented in rank order for comparison.

Boars	Fertility in vivo				Seminal plasma protein from Sperm-Rich Fraction				
	Pregnancy rate (%)	Farrowing rate (%)	Total piglets born (mean ± SE)	Fertility Index (mean ± SE)	Total protein concentration (mg/mL)	Total protein per AI dose (mg/AI dose)	D-PSP-I abundance (re/µg of total protein)	D-PSP-I concentration (re/mL)	D- PSP-I per AI dose (re/AI dose)
R-2	98 ^{x}	98 ^{x}	11.7 ± 0.4 ^{ab}	11.4 ^{a}	20.0 ^{c}	72	0.89	3529 ^{a}	13276
Y-2	91 ^{xy}	89 ^{xy}	12.0 ± 0.5 ^{a}	10.9 ^{ab}	19.4 ^{bc}	61	0.89	3432 ^{a}	10916
Pu-3	95 ^{xy}	91 ^{xy}	11.2 ± 0.5 ^{abc}	10.2 ^{ab}	31.8 ^{ab}	135	0.89	5643 ^{b}	25510
B-1	98 ^{x}	94 ^{xy}	10.7 ± 0.5 ^{abcd}	10.2 ^{ab}	36.7 ^{a}	126	0.90	6613 ^{b}	23152
R-3	94 ^{xy}	95 ^{xy}	10.9 ± 0.4 ^{abcd}	10.1 ^{ab}	35.9 ^{a}	139	0.85	6025 ^{b}	23260
G-2	93 ^{xy}	91 ^{xy}	10.1 ± 0.5 ^{abcd}	9.5 ^{ab}	36.4 ^{a}	144	0.88	6402 ^{b}	25188
B-3	93 ^{xy}	93 ^{xy}	9.6 ± 0.5 ^{cd}	8.8 ^{abc}	31.2 ^{ab}	110	0.89	5545 ^{b}	20505
R-1	86 ^{yz}	84 ^{yz}	10.0 ± 0.4 ^{bcd}	8.4 ^{bc}	19.0 ^{bc}	81	0.90	3434 ^{a}	15442
G-1	72 ^{z}	71 ^{z}	8.4 ± 0.6 ^{d}	6.0 ^{c}	30.9 ^{abc}	107	0.93	5719 ^{b}	19315
SE	N/A	N/A	-	±0.6	±6. 7	±35	±0.03	±1067	±4191
P	0.0003	0.0003	<0.001	<0.0001	0. 025	0.12	0.927	0.014	=0.14

Values in the table are least square means (LSM). $^{a-b}$: LSM with different superscripts within each column indicate differences due to boar ($P<0.05$).

SE= pooled standard errors of LSM. P= probability of main effect of boar. re= relative expression.

Table 4-3. Differences in seminal plasma proteins by fraction, using data from the nine boars evaluated. Seminal plasma samples were taken four times per boar during the 6.5 ±1 mo evaluation period.

Seminal Plasma Proteins	Seminal Plasma Fractions (9 boars)			
	Sperm-Peak	Sperm-Rich	Sperm-Free	± SE
Total Protein (mg/mL)	16 ^{y}	28 ^{x}	33 ^{x}	3.5
PSP-I (re/mL)	2827 ^{y}	4897 ^{x}	6307 ^{x}	648.0
D-PSP-I (re/mL)	2887 ^{y}	5069 ^{x}	6354 ^{x}	645.0

Values in the table are least square means (LSM). x-y: LSM with different superscripts within each row were different due to a seminal plasma fractions ($P<0.05$).
± SE: ± pooled standard errors of LSM.
re/mL= relative units per mL of seminal plasma.
PSP-I= Porcine seminal plasma –I protein
D-PSP-I= deglycosylated seminal plasma PSP-I

Table 4-4. Differences in seminal plasma proteins by fraction, using data from the two highest (R-2, Y-2) and two lowest (G-1 R-1) fertility boars. Seminal plasma samples were taken 4 times during the 6.5 ± 1 mo evaluation period.

Seminal Plasma Proteins	Seminal Plasma Fractions (Highest and Lowest fertility boars)			
	Sperm-Peak	**Sperm-Rich**	**Sperm-Free**	**± SE**
Total Protein (mg/mL)	14 [b]	22 [a]	27 [a]	2.8
PSP-I (re/mL)	2535 [b]	3891 [ab]	5203 [a]	716.0
D-PSP-I (re/mL)	2530 [b]	3975 [ab]	5300 [a]	754.0
AWN-1 (re/mL)	1636 [b]	2391 [ab]	2903 [a]	539.0
OPN-9 (re/mL)	309 [b]	489 [a]	645 [a]	65.0
OPN-12 (re/mL)	348 [b]	503 [a]	645 [a]	68.0
OPN-70 (re/mL)	515 [b]	731 [ab]	906 [a]	113.0

Values in the table are least square means (LSM). [a-b]: LSM with different superscripts within each row were different ($P<0.05$).
± SE: ± pooled standard errors of LSM.
re/mL: relative units per mL of seminal plasma; PSP-I: Porcine seminal plasma –I protein; D-PSP-I: deglycosylated seminal plasma PSP-I proteins; OPN: Osteopontin 9, 12 and 70 kDa molecular weight respectively.

Table 4-5. Differences between the Highest (R-2, Y-2) and Lowest (G-1, R-1) fertility boars in the seminal plasma proteins of the Sperm-Rich fraction.

Seminal Plasma Proteins	Fertility ranking	
	Highest	**Lowest**
Total protein concentration	20 ± 5	25 ± 5
AWN-1 concentration (re/mL)	1997 ± 397[a]	3021 ± 397[b]
Total protein (mg/AI dose)	67 ± 13	94 ± 13
AWN-1 (re/AI dose)	6926 ± 1509 [a]	11696 ± 1509 [b]
OPN-9 (re/AI dose)	1477 ± 215 [a]	2509 ± 215 [b]
OPN-12 (re/AI dose)	1541 ± 242 [a]	2580 ± 242 [b]
OPN-70 (re/AI dose)	3971 ± 737	3708 ± 737
Total protein/AI dose	3.40 ± 0.46	3.79 ± 0.46

Values in the table are least square means (LSM) ± pooled standard errors (SE) of LSM.
[a-b]: LSM with different superscripts within each row were different due to fertility ranking ($P<0.05$).
re/AI dose: relative units per artificial insemination dose.
OPN: Osteopontin 9, 12 and 70 kDa molecular weight respectively.

Table 4-6. Mean relative protein abundance (units/mg total protein) in seminal plasma in the Sperm-Rrich fraction of the two lowest and the two highest fertility boars for the four times analyzed within each boar.

Protein[1]	Fertility ranking			
	Lowest		Highest	
	G1	R1	R2	Y2
1	11.97 ± 0.86	11.86 ± 0.86	11.99 ±0.86	11.12 ± 0.86
2	10.35 ± 1.65	12.00 ± 1.65	12.71 ± 1.22	11.22 ± 1.57
3	10.45 ± 0.72	12.19 ± 0.88	11.60 ± 0.72	13.43 ± 1.11
4	14.03 ± 1.30	10.58 ± 1.02	9.95 ± 0.79	10.80 ± 1.83
5	12.01 ± 0.55	11.00 ± 0.67	10.89 ± 0.55	13.00 ± 1.23
6	12.63 ± 0.78	10.83 ± 0.95	11.84 ± 0.78	10.92 ± 1.75
7	**12.60 ± 0.83[a]**	**8.54 ± 0.83[b]**	**12.98 ± 1.28[a]**	**12.61 ± 1.00[a]**
8	11.12 ± 0.63	11.84 ± 0.82	10.28 ± 1.03	11.97 ± 1.46
9	13.72 ± 1.04	9.76 ± 1.28	10.58 ± 1.28	9.91 ± 1.62
10	10.28 ± 1.59	9.02 ± 1.32	12.95 ± 1.32	11.19 ± 1.59
11	12.36 ± 0.87	9.17 ± 1.35	11.46 ± 0.87	10.89 ± 1.35
12	9.35 ± 1.19	10.30 ± 1.43	11.21 ± 1.19	12.23 ± 1.19
13	11.28 ± 0.64	11.54 ± 0.64	12.61 ± 0.64	12.17 ± 0.64
14	11.01 ± 1.30	8.70 ± 2.05	11.57 ± 2.07	10.06 ± 1.62
15	12.20 ± 0.77	10.36 ± 0.77	11.14 ± 0.77	12.75 ± 0.77
16	12.70 ± 1.05	10.20 ± 1.05	10.76 ± 1.29	9.50 ± 2.37
17	**13.34 ± 0.82[a]**	**7.97 ± 1.00[b]**	**12.01 ± 0.82[a]**	**12.44 ± 1.26[a]**
18	11.13 ± 1.55	11.25 ± 1.55	12.45 ± 2.37	9.74 ± 1.55
19	12.57 ± 0.80	11.00 ± 0.82	12.37 ± 0.82	11.56 ± 0.80
20	12.20 ± 0.83	11.96 ± 1.00	11.83 ± 0.83	10.09 ± 0.83
21	11.79 ± 1.04	10.33 ± 1.04	11.98 ± 1.04	11.96 ± 1.04
22	**12.32 ± 1.11[a]**	**6.10 ± 0.83[b]**	**11.78 ± 0.83[a]**	**14.93 ± 1.11[a]**
23	11.90 ± 2.36	11.74 ± 2.36	9.67 ± 1.49	11.22 ± 1.49
24	**10.80 ± 1.00[ab]**	**12.67 ± 1.21[a]**	**13.85 ± 1.21[a]**	**8.48 ± 1.00[b]**
25	12.75 ± 2.76	9.21 ± 2.04	10.84 ± 3.85	12.44 ± 2.12
26	10.17 ± 0.82	10.55 ± 0.99	12.97 ± 0.82	11.38 ± 0.82
27	12.81 ± 0.57	11.57 ± 1.35	10.77 ± 0.73	10.30 ± 0.93
28	10.35 ± 2.59	11.24 ± 2.15	12.94 ± 2.15	9.40 ± 2.59
29	12.16 ± 1.03	10.81 ± 1.03	11.81 ± 1.03	11.80 ± 1.03
30	12.77 ± 1.22	10.67 ± 1.20	12.22 ± 1.22	11.51 ± 1.20
31	10.50 ± 0.93	11.51 ± 0.76	12.08 ± 0.76	13.42 ± 1.70
32	11.87 ± 0.89	11.22 ± 0.94	12.94 ± 0.94	11.24 ± 1.97
33	11.47 ± 2.26	9.52 ± 2.26	10.41 ± 1.84	12.09 ± 2.22
34	10.69 ± 3.60	7.69 ± 6.81	8.00 ± 3.75	10.49 ± 4.87
35	11.89 ± 0.38	11.28 ± 0.38	11.35 ± 0.38	11.55 ± 0.38
36	12.69 ± 0.87	11.62 ± 0.72	11.96 ± 0.72	11.40 ± 0.87
37	13.47 ± 1.06	11.49 ± 0.83	10.63 ± 0.82	11.83 ± 0.68
38	12.06 ± 1.87	11.80 ± 1.17	10.59 ± 1.85	12.58 ± 1.46
39	12.42 ± 0.76	12.03 ± 0.76	12.01± 0.76	11.33 ± 0.76
40	11.59 ± 0.43	11.56 ±0.43	10.93 ± 0.52	12.77 ± 0.43
41	11.20 ± 0.73	11.74 ± 0.88	11.23 ± 0.73	12.93 ± 0.73
42	11.55 ± 1.07	11.04 ± 1.07	14.34 ± 1.63	11.43 ± 1.07

[a,b] Different letters within rows indicate differences between means due to boar ($P<0.05$). [1]= The protein number corresponds to the numeric identification for the protein spots shown in Figure 4-5.

Figure 4-2. Representative immunoblot of a 1-D SDS-PAGE gel using a specific PSP-I antiserum. PSP-I immunoactivity of glycosylated (odd numbered lanes) and deglycosylated (even numbered lanes) seminal plasma protein samples from five boars (B-1, G-1, R-1, R-2, Y-2). A pooled seminal plasma sample was run as an Internal Control in all gels to allow estimation of relative abundance across multiple gels.

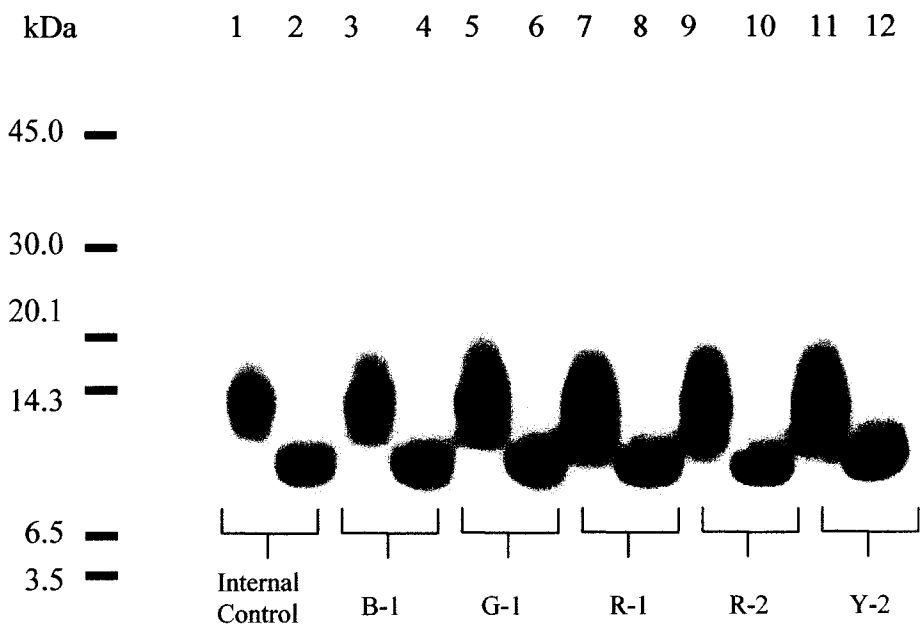


Figure 4-3. Representative immunoblot of a 1-D SDS-PAGE gel using a specific AWN-1 antiserum and 5 mg per lane of glycosylated seminal plasma protein. Samples were from the 3rd collection from the two highest (R-2, Y-2) and the two lowest (G-1, R-1) fertility boars and from the Sperm-Peak (SP), Sperm-Rich (SR) and Sperm-Free (SF) fractions. A pooled seminal plasma sample (C) was run as an Internal Control in all gels to allow estimation of relative abundance across multiple gels.

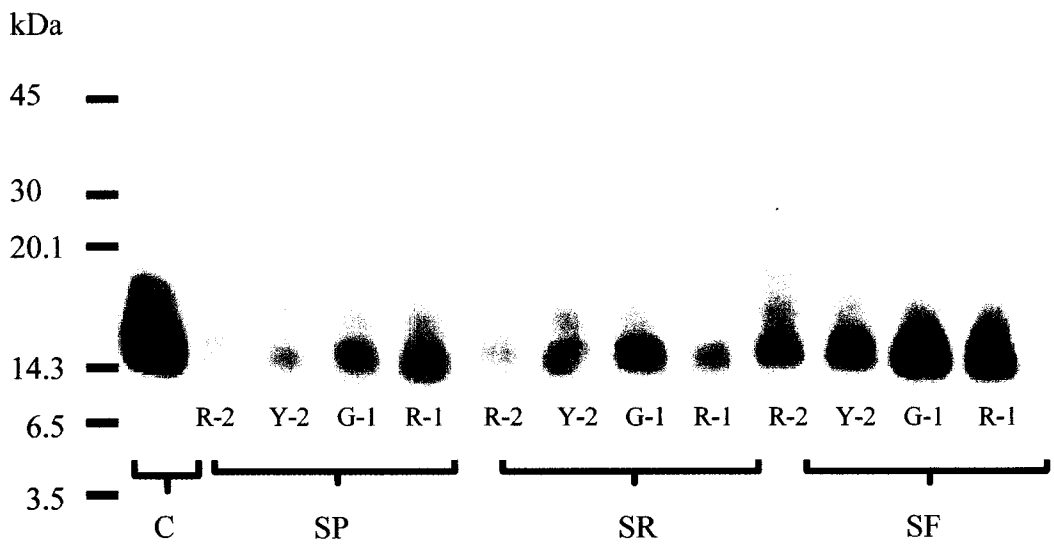


Figure 4- 4. A representative immunoblot of one dimensional SDS-PAGE gel using a specific osteopontin antiserum, and 25 mg per lane seminal plasma protein samples from the two highest (R-2, Y-2) and the two lowest (G-1, R-1) fertility boars from the Sperm-Free fraction. Sow milk osteopontin served as a positive control (lane 6) and A pooled seminal plasma sample (C; lane 5) was run as an Internal Control in all gels to allow estimation of relative abundance across multiple gels.

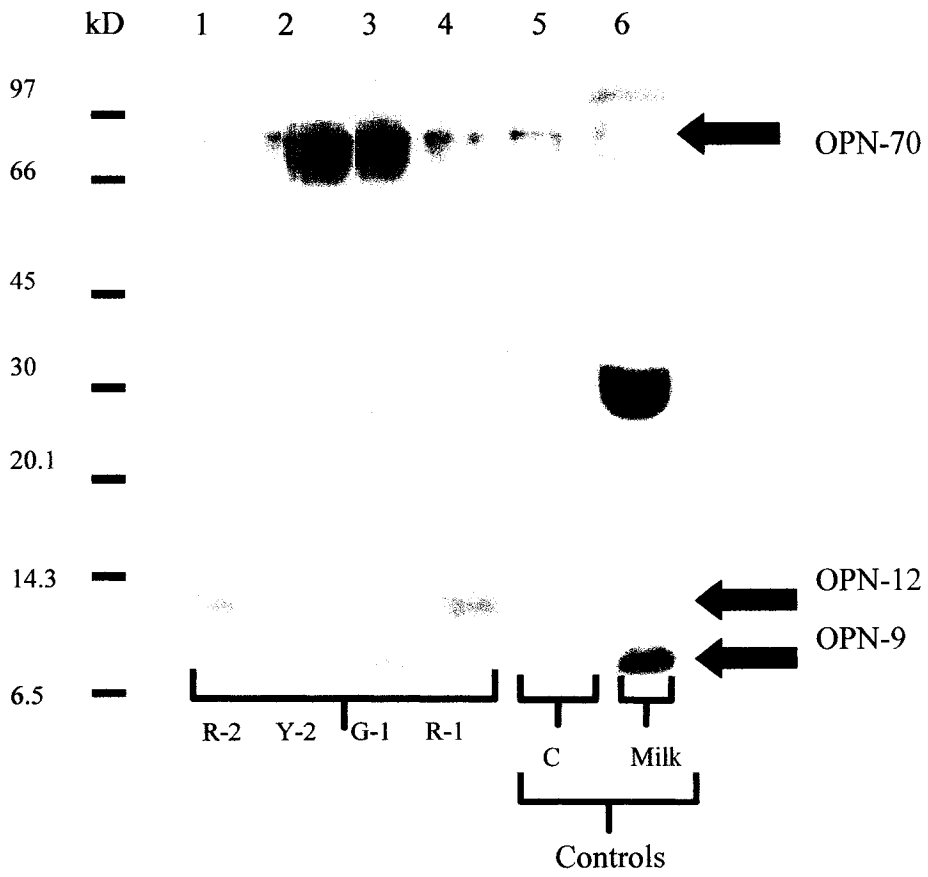


Figure 4-5. A representative 12% (w/v) 2-D SDS-PAGE gel showing seminal plasma proteins identified from the Sperm-Peak fraction of boar R-1. The 42 spots analyzed by the Imagemaster software are numbered 1 to 42, and correspond to the proteins for which relative abundance is presented in Table 4-6.

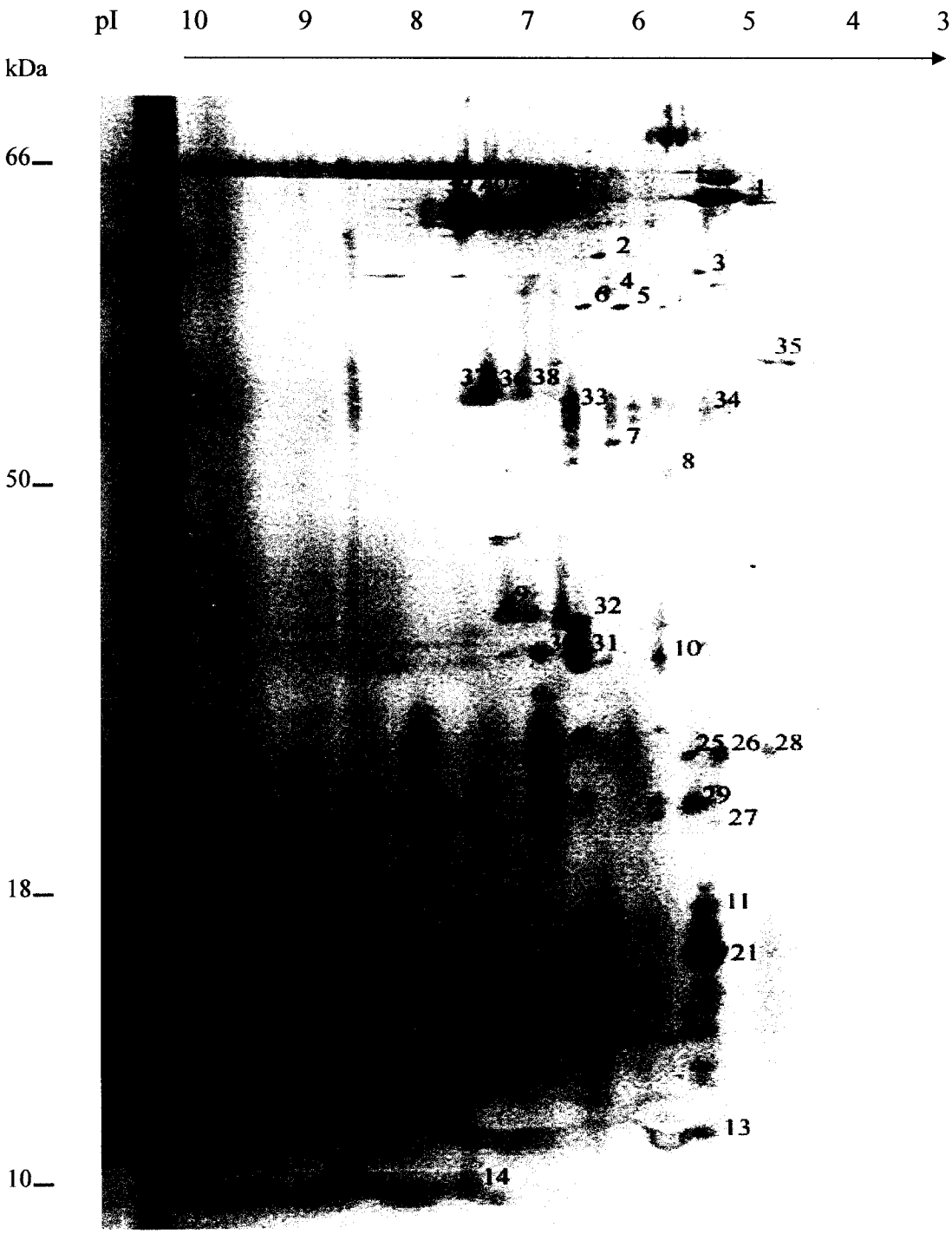
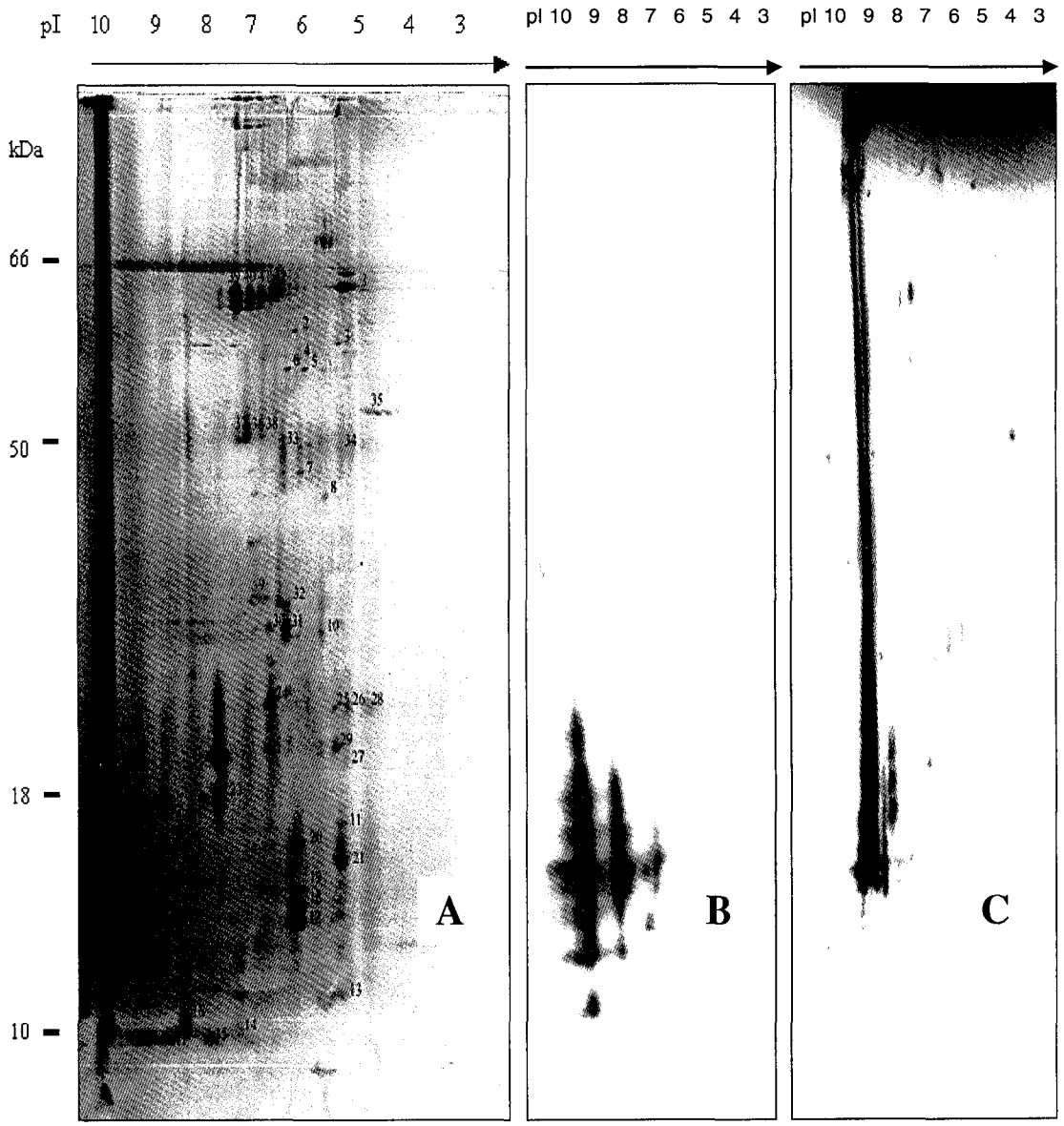


Figure 4-6. 4-6A: The representative 12% (w/v) 2-D SDS-PAGE gel showing seminal plasma proteins identified from the Sperm-Peak fraction of boar R-1 in Figure 4.5. 4-6B: Western blot using PSP-1 antibody on a blot transferred from a duplicate gel to that shown in Figure 4-6A. 4-6C: Western blot using AWN-1 antibody on a blot transferred from a duplicate gel to that shown in Figure 4-6A.



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CHAPTER FIVE

CHANGES IN SPERM MOTILITY OF EXTENDED SEMEN AS AN INDICATOR OF BOAR FERTILITY

5.1 INTRODUCTION

The use of AI in the swine industry has increased tremendously in recent years, dramatically improving the efficiency of pork production and increasing the demand for good quality processed semen. Currently, routine semen evaluation commonly used during semen processing in AI centers (sperm concentration, morphology, viability, and motility) only detects male reproductive disorders that result in low fertility. However, these parameters are not useful for predicting relative fertility in healthy boars with ejaculate quality that meets normal industry standards (>70 % motility and <30% abnormal sperm) (Flowers, 1997), even though the productivity of these boars may be substantially different (Ruiz-Sánchez et al., 2006). This is especially important when low sperm doses (<2.5 billion sperm/dose) are used for AI (Tardif et al., 1999; Watson and Behan, 2002; Mezalira et al., 2005; Ruiz-Sánchez et al., 2006).

Conventional AI (intra-cervical insemination) commonly uses high sperm numbers (>2.5 x 10^9 sperm per dose) to ensure fertility levels similar to natural mating. This practice does not optimize the efficient use of high genetic index,high fertility boars and allows less fertile boars (undetected due to sperm number compensation) to remain in production for a longer period of time (Ruiz-Sánchez et al., 2006). Also due to major disease outbreaks in the last decade (Foot and mouth disease, Classical swine fever, bird flu, etc.), bans on animal movement in affected regions have been established as a biosecurity measure, increasing the need for more efficient use of boars in order to cover all breeding demands. As a result, new AI techniques such as post-cervical (Watson and Behan, 2002; Roca et al., 2006) and

deep intra-uterine insemination (Martínez et al., 2001; Vázquez, 2003; Vázquez et al., 2005; Mezalira et al., 2005) have been developed, to reduce the number of sperm per AI dose without affecting reproductive outcomes. Unfortunately, variable results have been obtained with these insemination techniques, which are perhaps attributable to differences in semen quality and boar fertility (Mezalira et al., 2005).

Currently, improved efficiency of boar reproduction is limited by the lack of good indicators of boar fertility and semen quality. The use of low sperm AI doses to determine relative boar fertility *in vivo* is very reliable (Ardón et al., 2003; Ruiz-Sánchez et al., 2006), but generally impractical at a commercial level due to its impact on productivity of the breeding herd. The complexity of the fertilization process has prompted the use of sophisticated *in vitro* tests to evaluate sperm characteristics directly related to fertility. Techniques such as zona pellucida (ZP) (Ivannova and Mollova, 1993) and oocyte penetration rate (Berger and Parker, 1989; Gadea et al., 1998; Gadea and Matas, 2000) have been used to successfully identify fertile vs. sub-fertile boars and/or ejaculates. Using standardized oocyte *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) techniques, Xu et al. (1998) and Ruiz-Sánchez et al. (2006) reported that IVM and IVF could be useful as a complementary test to identify relatively sub-fertile boars. Unfortunately, this method is expensive and time-consuming and likely to be impractical as a routine procedure in commercial AI centers.

In a recent study by Ruiz-Sánchez et al. (2006), the motility of extended semen at Days 7 and 10 of storage correlated with litter size ($r=0.42$ and 0.37, respectively; $P<0.05$) and farrowing rate ($r=0.36$ and 0.30, respectively; $P<0.05$), confirming previous reports of positive correlations with *in vivo* fertility and sperm motility (Juonala et al., 1998; Sutkeviciene et al., 2005). Furthermore, Xu et al. (1996a) found differences among boars in the motility of their extended semen at Day 7, and a very high correlation ($r=0.90$, $P<0.001$) between this characteristic and the oocyte penetration rate *in vitro* of sperm processed from their fresh ejaculates. Therefore, we hypothesized that the different rates of motility loss in extended semen over a period

of 7 to 10 days would reflect differences in relative fertility in young boars. This would provide a practical and economical fertility test that could be used at commercial level.

When considering the optimal *in vitro* tests of semen quality that could be associated with differences in sperm motility during storage, several of the IVM and IVF techniques reported previously (Ding and Foxcroft, 1992; Ding et al., 1992; Rath, 1992; Ding et al., 1994ab; Zak et al., 1997) were based on protocols to assess oocyte quality and embryo production potential. Therefore, they had inherently different objectives than the assessment of potential boar fertility. As discussed previously (Ruiz-Sánchez et al., 2006), one of the factors that could optimize the IVM-IVF technique for assessing relative boar fertility would be the use of a fixed total number of sperm per oocyte after *in vitro* capacitation, rather than correcting sperm numbers used for IVF to account for differences in the number of motile sperm after *in vitro* capacitation. This would seem appropriate, given that differences in sperm motility after capacitation *in vivo* are not corrected in the pre-fertilization environment of the oviduct. Also, since there are certain sperm characteristics that can be compensated by increasing sperm numbers available for fertilization (Saacke et al., 2000), this practice may give a less accurate indication of relative boar fertility. In contrast, if equal numbers of total sperm are used for IVF, all the boars will be tested in a more representative manner. As a result, sperm quality variation among boars affecting the efficiency of fertilization will be more readily identified and result in better correlations between *in vivo* and *in vitro* fertility. Therefore, we hypothesized that for the IVF assessment used in the present study, if total sperm numbers per oocyte were not corrected for differences in motility after *in vitro* capacitation, this would reveal differences of relative boar fertility and allow significant associations with the loss of motility of stored semen to be evaluated.

The principal objectives of the present study were: 1) to determine the relative changes in motility of stored extended semen collected from a population of young

boars whose ejaculates would be considered acceptable for use in AI programs, based on characteristics typically measured in commercial AI centers; 2) to identify sub-populations of potentially Low or High fertility boars, based on our previous results linking differences in motility in extended semen to relative boar fertility *in vivo* (Ruiz-Sánchez et al., 2006); 3) to determine the relative fertility of these sub-populations of boars using IVF of homologous, zona-intact, *in vitro* matured pig oocytes in combination with different sperm:oocyte ratios that were not corrected for sperm motility after *in vitro* capacitation.

5.2. MATERIALS AND METHODS

5.2.1. Boar selection

Experiments were conducted at the University of Alberta in collaboration with Alberta Swine Genetics Corporation (ASGC) (Nisku, AB, Canada). Potential candidate boars for this study were identified during a 5 to 6-wk quarantine period prior to entering the stud. During this time, at least three consecutive ejaculates from 15 boars of 24 to 28 wk of age were evaluated. Complete ejaculates were collected from each boar by the gloved hand technique and transported in a pre-warmed styrofoam box to the University of Alberta semen evaluation lab (30 ± 15 min). The same experienced technician performed the following semen evaluations:

1) Total volume (mL) was estimated by weighing the complete ejaculate and assuming a density of 1 g per mL.

2) Sperm morphology was evaluated using an eosin-nigrosin vital stain (Minitube of America Inc. Verona, WI) under a microscope (Olympus CH30, Japan) fitted with a 40x phase contrast lens. A smear was prepared by mixing a drop of semen with a drop of stain on a preheated glass slide. The smear was air-dried, at least 200 sperm cells were evaluated, and the percentage of spermatozoa with abnormal heads, cytoplasmic droplets (both distal and proximal), abnormal tails and total

number of abnormal sperm were calculated. If a high proportion of detached heads were found, a second smear was prepared in order to confirm the results.

3) Sperm concentration was measured at the collection site using a calibrated spectrophotometer (Spermacue, Minitube of America).

4) Progressive motility of raw semen on the day of collection (Day 0) was evaluated at 37 °C using a microscope with a final magnification of 200x, screening at least five different fields. An estimate of progressive motility was subjectively scored to the nearest 5%.

5) Finally, the ejaculate was diluted (1:10) with Beltsville Thawing Solution containing antibiotics (BTS, Minitube of America) and stored at 17 °C in 3 mL aliquots (5 mL glass tubes) for further motility evaluations. On Days 3, 7, and 10, a sample of diluted semen (3 mL aliquot) was gently mixed and incubated at 37 °C for 20 min in a multiblock heater (H22025-1A Lab-Line Instruments, Inc, Melrose Park, IL). Prior to examination 200 μL of caffeine solution (15 mg/mL caffeine (w/v)) were added to the sample and motility assessment was carried out following the same protocol described above.

Boars were only considered to be candidates for this experiment if they had more than 80 % of normal sperm and greater than 80 % motility in raw semen. Based on sperm motility of stored semen at Days 7 and 10, five boars were characterized as Low motility (L), and four as High motility (H), and ejaculates from these boars was used for fertility evaluation *in vitro*. However, due to logistical problems in the IVM/IVF evaluation, two of the selected High motility boars were removed from the trial. Data on the routine semen evaluation of the 15 boars during the quarantine period, and the identity of the High and Low motility boars, are presented in Table 5-1.

5.2.2. Boar fertility in vitro evaluation

At the time of the *in vitro* evaluations, the selected boars were between 30 and 38 wk of age and were collected once a week during a three-week period using the gloved hand technique. A complete single ejaculate was collected into a pre-warmed collection vessel lined with a collection bag with integrated filter. A sample of 6 mL from each ejaculate was transferred into a pre-warmed 15mL falcon tube with 0.5 mg Licomycin and 1 mg spectinomycin (Linco-Spectin, Pharmacia Animal Health, Orangeville, ON, Canada) and transported immediately to the Swine Research and Technology Center AI laboratory (University of Alberta, Edmonton, AB, Canada) in a pre-warmed styrofoam box. Each ejaculate sample was processed as follows:

1) Sperm morphology was evaluated using an eosin-nigrosin vital stain as described above.

2) Acrosome integrity was evaluated by mixing 0.9 mL of a formal citrate solution (2.9 mg/mL sodium citrate and 4 μL/mL 40% formaldehyde) with 0.1 mL of semen in a 2-mL vial and one drop of the mixture was then observed under a microscope (Olympus CH30, Japan) fitted with a 40x and 100x phase contrast lens. At least 200 cells were evaluated and the percentage of cells with acrosomal damage was calculated.

3) Progressive motility on the day of collection was evaluated at 37 °C as described previously.

4) pH was measured using pH-indicator strips (colorpHast® pH 6.5-10.0, EMD Chemicals Inc. Gibbstown, NJ).

5) For further motility and pH evaluations, semen was extended by adding 1 mL of ejaculate to 9 mL of Beltsville Thawing Solution containing antibiotics (BTS, Minitube of America) and then stored at 17°C in aliquots of 3 mL (5 mL glass tubes). On Days 0, 3, 7, and 10, a sample of diluted semen (3 mL aliquot) was gently mixed

and placed for 20 min into a multi-block heater pre-warmed at 37°C. Caffeine was added to the sample prior to examination and motility assessment was carried out following the same protocol used above.

6) Finally, the remaining 5 mL of the ejaculate sample was kept at room temperature (20°C) for approximately 18 h after collection for *in vitro* evaluation. A 6-mL sample of sperm rich fraction from a mature boar of proven fertility was collected during the three IVF periods and used as an internal control for the IVM-IVF assay.

5.2.3. In vitro fertilization evaluation

Semen quality from selected boars was evaluated using a previously described IVF procedure (Ruiz-Sánchez et al., 2006) with the modifications outlined below.

5.2.3.1. Culture media.

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis. MO, USA). Oocyte wash medium was prepared with 0.100 μg/mL PVA, 6.663 μg/mL sodium chloride, 0.168 μg/mL sodium hydrogen carbonate, 0.239 μg/mL potassium chloride, 1.426 μL/mL sodium lactate, 0.041 μg/mL sodium hydrogen phosphate (monobasic), 0.102 μg/mL magnesium chloride (hexahydrate), 2.186 μg/mL sorbitol, 2.383 μg/mL HEPES, 0.022 μg/mL sodium pyruvate and 0.294 μg/mL calcium chloride. Oocyte maturation medium consisted of North Carolina State University (NCSU) 23 medium (Petters and Wells, 1993) supplemented with 2.5 μL/mL β-mercaptoethanol (10 mM stock solution) and 0.1 μg/mL cysteine.

The maturation medium was also supplemented with 10% (v/v) porcine follicular fluid (pFF) obtained from clear follicles 5 to 8 mm diameter which was centrifuged at 1680 x g for 30 min, filtered with a 0.22 μm syringe filter and stored at –15 °C until use.

Fertilization medium was prepared using a modified TRIS buffered medium (2.42 mg/mL TRIS base, 6.612 mg/mL sodium chloride, 0.22 mg/mL potassium

chloride, 1.98 mg/mL glucose, 0.55 mg/mL sodium pyruvate, and 1.102mg/mL calcium chloride), supplemented with 0.192 mg/mL caffeine and 1.0 mg/mL BSA (A7888).

Sperm-free medium consisted of North Carolina State University (NCSU) 23 medium containing 75 μg/mL penicillin, 50 μg/mL streptomycin and supplemented with 4 mg/mL BSA (A8022) immediately before use.

5.2.3.2. Oocyte collection and in vitro maturation

Ovaries with well-developed Graafian follicles were collected from prepubertal gilts immediately after slaughter through the cooperation of staff of a local abattoir (Olymel, Red Deer, Alberta, Canada). To minimize temperature changes and bacterial contamination, the ovaries were transported to the laboratory within 90 min of collection in thermos flasks containing sterile physiological saline at 30°C with added antibiotics (75 μg/mL of penicillin G potassium; 50 μg/mL of streptomycin sulphate). Ovaries were then washed three to four times with pre-warmed saline solution before aspiration of follicles. Cumulus-oocyte-complexes (COC's) were aspirated from clear follicles with a diameter of 3 to 6 mm using a 10 mL syringe fitted with an 18 ga needle. COC's were washed three times with pre-warmed oocyte wash medium (PVA-HEPES). Only COC's with uniformly dark cytoplasm and several compact layers of cumulus cells were selected and washed twice with oocyte wash medium, and then washed once with oocyte maturation medium. Approximately 50 COC's were then transferred to a Falcon culture dish (Becton Dickinson Labware, Mississauga, ONT) containing 0.5 mL of oocyte maturation medium supplemented with 10 IU/mL hCG (Chorulon, Intervet, Canada, Whitby, ON), 10 IU/mL PMSG (Folligon, Intervet) and 1 mM db cAMP (N6,2'-0-Dibutyryladenosine 3'-5' cyclic), and incubated at 39 °C in an atmosphere of 5% (v/v) CO_2 in air. After 20 to 22 h, oocytes were washed and transferred to oocyte maturation medium, without hormones, and incubated for an additional 20 to 24 h.

5.2.3.3. Sperm capacitation

As mentioned previously, the remaining 5 mL of the ejaculate sample were kept at room temperature (20 °C) for approximately 18 h after collection for use in the *in vitro* fertilization evaluation test. A 2 mL aliquot from the middle of the settled sperm layer was then transferred into a 15 mL Falcon tube, and diluted with 8 mL sterile physiological saline and washed by centrifugation for 5 min at 1000 x g. The supernatant was discarded and the sperm pellet re-suspended in 9 mL physiological saline. This washing procedure was repeated twice. The supernatant was then discarded and the sperm pellet re-suspended in fertilization medium to a concentration of 4 x 10^8 total sperm/mL and incubated at 39 °C under an atmosphere of 5% (v/v) CO_2 in air for 150 min.

5.2.3.4. In vitro fertilization

After in vitro maturation, COC's with fully expanded cumulus cells were selected and washed twice in fertilization medium. Ten oocytes were transferred to each well of a 4-well culture dish (Nucleon 176740, Mississauga, ON) containing 0.95 mL of fertilization medium per well and incubated pending IVF.

After capacitation, sperm motility was recorded and concentration adjusted to provide three different sperm:oocyte ratios of 25 x 10^3, 50 x 10^3 and 75 x 10^3 total sperm per oocyte. Sperm and oocytes were incubated at 39°C under 5% (v/v) CO_2 in air. After 6 h, oocytes were washed three times with sperm free medium. They were then transferred into a 4-well culture dish containing sperm free medium and cultured for another 6 h ± 30 min at 39 °C under 5% (v/v) CO_2 in air. Finally, 12 h after fertilization, the oocytes were transferred from the incubator to a refrigerator for storage overnight at approximately 5°C until examination. The internal IVF control sample was evaluated at the 75 x 10^3 total sperm:oocyte ratio throughout the three replicates. A minimum average penetration rate of 40% with semen from the IVF control sample was required in order that the IVM/IVF assay be deemed functional.

5.2.3.5. Sperm penetration assessment

Oocytes were mounted on slides and fixed for at least 48 h in slide fixing solution (1:3 acetic acid: ethanol), stained with lacmoid, and examined 1 d later under a phase-contrast microscope (Dialux 20 EB Leitz Wetzlar, Germany) at 200 x and 400 x magnification. Penetration rate (percent of mature oocytes penetrated), average number of sperm penetrated per mature oocyte (including all penetrated sperm forms, from slightly swollen sperm head(s) to a full-sized male pronuclei), monospermy rate, polyspermy rate, male pronuclear formation rate (MPN-f) (estimated as the percentage of penetrated oocytes with at least one male pronucleus), and potential embryo production rate (% of penetrated oocytes with both a female and a single male pronucleus) were determined as reported previously by Xu et al. (1998) and Ruiz-Sánchez et al. (2006).

5.2.4. Statistical analysis

A total of 3,500 matured oocytes were evaluated, comprising three replicates of the IVF evaluations of semen from the same boars (IVFtime = each IVF evaluation). Differences between High and Low motility groups for IVF characteristics were, therefore, analyzed as a repeated measures design using the mixed procedure of Statistical Analysis System (SAS version 8.2, SAS Institute Inc. Cary, NC, USA). The fixed effects were IVFtime (3 levels), motility groups (n=2), sperm:oocyte ratio (n=3), and their interactions. Due to a significant IVFtime by motility group interaction, differences among treatments were compared within each sperm:oocyte ratio using a repeated measures analysis of the mixed model procedure that included IVFtime (3 levels), motility groups (n=2), and their interactions. Differences among the motility groups in the routine semen evaluation characteristics and motility of stored semen were also analyzed using a repeated measures analysis of the mixed procedure of Statistical Analysis System. The fixed effects were IVFtime (3 levels), motility groups (n=2), and their interaction. In all statistical models, the Kenward-Roger option was used to calculate the denominator degrees of freedom. The variance-covariance matrix was chosen for each statistical model by an interactive process, wherein the best fitting model was based on Schwarz's Bayesian criteria.

Least square means (LSM) and standard errors (SE) were generated and separated using a pdiff adjusted by Tukey option for significant fixed effects. All percentile data were subjected to arcsine transformation before analysis. All data are presented as LSM ± SE of LSM.

The INSIGHT procedure (SAS, 2003) was used to examine relationships between the motility of stored semen at Days 0, 3, 7 and 10 (pre-selection data) and IVF outcomes. For this analysis, only the 75 x 10^3 sperm:oocyte ratio data were used, as for this data no significant IVFtime by motility group interaction, and no significant difference in IVFtime were found in the IVF parameters evaluated.

5.3 RESULTS

5.3.1. Pre-selected boars' semen evaluation

The pre-selection semen evaluation parameters of percent normal sperm, total sperm per ejaculate, percent motility of raw semen, and motility at Day 0 or Day 3 of extended semen storage were not different for High and Low motility boars (Table 5-2a). By definition, Low motility boars had higher rates of motility loss than the High motility boars, from Day 7 (P=0.06) through Day 10 (P=0.01), as shown in Figure 5-1a.

5.3.2. In vitro fertility evaluation period

5.3.2.1. Routine optical semen evaluation

During the *in vitro* fertilization evaluation period there were no differences between High and Low motility groups in total sperm per ejaculate, normal morphology (%), acrosomal damage (2.9±0.79 vs 2.4±0.50 %, respectively), pH (7.2±0.12 vs 7.2±0.08, respectively) and motility (%) of raw semen (Table 5-2b). Even though the motility degradation trends observed for the boar groups were similar to those seen during the selection period, no significant differences between the High

and Low motility groups were detected at any storage time (Figure 5-1b). Furthermore, motilities of extended semen changed with IVFtime, but no significant interactions between motility group and IVFtime were seen in any of the characteristics examined.

5.3.2.2. *In vitro* fertilization evaluation

Significant interactions were established between motility groups and IVFtime with the 25 x 10^3 and 50 x 10^3 sperm:oocyte ratios for certain *in vitro* parameters (data not shown), including penetration rate at the 25 x 10^3 sperm:oocyte ratio (Table 5-3). However, no interactions among any of the IVF parameters were found when the 75 x10^3 sperm:oocyte ratio was evaluated, and differences among the motility groups in penetration rate, average number of penetrated sperm per oocyte, monospermy and polyspermy rate, number of MPN, MPN-f rate and potential embryo formation rate are presented in Table 5-4. No differences over IVFtime were observed for this sperm:oocyte ratio in any of the parameters evaluated.

5.3.3. Correlations between extended semen motility and IVF assay

To identify if the motility of stored-extended semen could be useful to predict relative boar fertility *in vitro*, independent relationships between extended semen motility values at Days 0, 3, 7 and 10 obtained during the pre-selection period and parameters obtained *in vitro* were analyzed. For this analysis, data from the 75 x 10^3 total sperm:oocyte ratio was used because it did not present any IVFtime by Motility group interactions and no differences due to IVFtime existed. Motility at Day 7, during the pre-selection evaluation, was highly correlated with penetration rate (r=0.84, P=0.009), monospermy rate (r=0.86, P=0.01), MPN formation rate (r=0.89, P=0.03), and potential embryo production rate (r=0.70, P=0.07), as was motility at Day 10 (r=0.88, P 0.01; r=0.84, P=0.02; r=0.85, P=0.01; r=0.77, P=0.04, respectively).

5.4. DISCUSSION

As earlier studies have shown, once estimates of sperm motility and normal morphology in fresh semen exceed 60 %, these characteristics are not useful predictors of boar fertility when commercial AI doses are used (2 billion or more sperm per AI dose) (Flowers, 1995; Berger and Parker 1989; Flowers, 1997; Flowers and Tuner, 1997; Xu et al., 1998; Popwell and Flowers, 2004). However, productivity of these boars may still be substantially different (Flowers, 1995), particularly when lower sperm AI doses (1.5 to 0.3 billion sperm per dose) are used (Tardif et al., 1999; Watson and Behan, 2002; Ruiz-Sánchez et al., 2006). It is, therefore, important to emphasize that the ejaculate quality of all the boars included in this experiment exceeded normal industry standards (>80% progressive motility and >85% morphologically normal sperm). Furthermore, there were no differences between the motility groups ($P>0.05$) in the routine evaluation characteristics of raw semen during both the pre-selection and IVF evaluation periods as presented in the results section.

The differences detected among boars in sperm motility of extended stored semen is consistent with data from earlier studies in our laboratory, where differences among boars were reported for the percentage of sperm motility of stored semen at Day 7 (Xu et al., 1996a; Ruiz-Sánchez et al., 2006), Day 8 (Xu et al., 1998) and Day 10 (Ruiz-Sánchez et al., 2006). Furthermore, the primary results of this experiment indicate a significant difference in fertility *in vitro* between motility groups, as well as a high correlation between fertility *in vitro* and motility of extended semen at Days 7 and 10 of storage. These results are consistent with reported positive correlations between motility at Day 7 and *in vivo* (Juonala et al., 1998; Sutkeviciene et al., 2005; Ruiz-Sánchez et al., 2006) or *in vitro* fertility (Xu et al., 1996a). Therefore, our results suggest that evaluation of semen collected immediately after the boars have been trained for AI may be predictive of subsequent relative boar fertility.

It is important to note that BTS extender (a short-term extender) was used in this study to avoid any confounding effects that a long-term extender could have on

the fertility evaluation. Given the inherent ability of long-term extenders to preserve sperm characteristics (Huo et al., 2002; Dubé et al., 2004; Vyt et al., 2004; De Ambrogi et al., 2006), they would seem less appropriate for use in this type of evaluation. This could be one of the reasons why Xu et al. (1998) could not find significant correlations between motility at Day 7 and fertility *in vivo* when using the Androhep (a long-term) extender. This contradicted their previous studies in which BL-1 (a short-term extender) was used and they found a significant correlation ($r=0.8989$; $P<0.0001$) between Day 7 motility and penetration rate (Xu et al., 1996a). Therefore, if a long-term extender is used, the storage time necessary to see similar effects may need to be longer than the 7 and 10 Days already used for BTS. Further investigations would be required to establish the appropriate storage period to use for each specific type of semen extender.

It should also be noted that during the IVF evaluation period, sperm motility in extended semen from all the boars was lower than that observed during the boar selection period. Factors that may have attributed to the lower motility of extended semen during this period could include:

1) The pH of the extender used during the first collection week which was higher than normal (pH 8.8 vs. 7.2 (Vyt et al., 2004; Medrano et al., 2005)). For subsequent weeks, the extender was prepared a few hours before use and the pH was verified. The motility of subsequent samples was better, but was still lower than that observed previously.

2) Given that smaller semen aliquots (6 mL in a 15-mL Falcon tube) were available for this portion of the study, it was more difficult to maintain sample temperature during transport to the laboratory. This did not represent a problem during the pre-selection period, as the complete ejaculates were taken to the lab and sample temperature was more readily maintained.

The presence of antibiotics associated with the IVF procedure did not appear to

affect sperm motility (data not shown). For future studies, pre-dilution of the ejaculate sample on the collection site will be recommended to maintain constant conditions for sperm motility assessment of stored semen. For the IVF evaluation of fertility, these factors were not an issue, as they follow the same protocol and conditions previously used for this technique were followed (Xu et al., 1996; Xu et al., 1998; Ruiz-Sánchez et al., 2006).

Earlier studies in our research group identified a high correlation between *in vitro* characteristics and measures of *in vivo* fertility (Xu et al., 1998). A subsequent study, using low sperm numbers per AI dose to identify fertility differences, demonstrated that boars that showed lower fertility *in vivo* were also found to have lower fertility *in vitro* (Ruiz-Sánchez et al., 2006). Based on this and the *in vitro* fertility data alone, our results have confirmed these earlier reports of differences in the relative fertility of boars normally classified as having acceptable ejaculate characteristics (motility and normal morphology higher than 70%) and these differences in relative fertility can be identified with appropriate IVM-IVF techniques.

In this study, the variation in IVM-IVF results over time could be due to differences in oocyte quality. Ovaries were obtained from a local abattoir where there was no control over the physiological state of the slaughtered gilts (i.e. nutrition, genotype, age, etc.), possibly affecting the quality of the oocytes week by week (Hyun et al., 2003; Hermansson et al., 2006). Athough there were significant differences in overall oocyte maturation rate between IVFtimes (56.13 ± 2.4^{a}, 65.3 ± 2.4^{b}, $64.8^{b}\pm2.4$ for IVFtimes 1, 2 and 3, respectively; $P<0.05$), the random allocation of expanded COC's to culture wells representing all sperm:oocyte ratios and sperm motility groups, ensures that these effects of IVFtime on oocyte maturation rate will not influence the assessment of relative sperm quality. Furthermore, the lack of significant correlation between penetration rate and oocyte maturation rate ($r^2=0.05$, $P=0.567$) suggests that expanded COC's selected for IVF were competent to undergo fertilization.

An interaction between IVFtime and motility groups was gradually eliminated as the number of sperm per oocyte increased, resulting in no significant interactions for any of the IVF parameters at the 75 $x10^3$ sperm:oocyte ratio. Examining the penetration rate data by motility group and IVFtime, the Low motility group showed the lowest penetration rates for all IVF times, even though during the second IVF assay there was no significant difference between motility groups when data for the 25 x 10^3 sperm:oocyte ratio was evaluated. As the number of sperm increased (25 x 10^3 to 75 x 10^3 sperm:oocyte ratios) the penetration rates of the Low motility boars did not increase at the same rate as the High motility boars, allowing the differences between the motility groups to become apparent with no IVFtime effect. This suggests that sperm traits of the Low motility boars cannot be fully compensated by increasing sperm numbers (Saacke et al., 2000). As shown in this experiment, inter-assay variation could affect the results depending on the sperm:oocyte ratio used, demonstrating the importance of examining different sperm:oocyte ratios. This allows an appropriate ratio to be identified, which is not affected by interassay variation, but is sensitive enough to detect the differences between boars.

Ruiz-Sánchez et al. (2006) reported significant differences between boars in *in vitro* penetration rate, and the penetration rates reported were higher (97±5 to 49±5%) than for the High and Low motility groups in the present study (66±3.5 to 35±2.2 %). The lower penetration rates in the present study could be due to several factors. Firstly, sperm from young boars tends to show lower penetration rates *in vitro* than sperm from older more mature boars. The boars in this experiment were 30 to 38 wks of age during the IVF evaluation period and the boars used in the previous experiment were evaluated over a longer period of time (from approximately 30 to 66 wk of age). Secondly, in contrast to previous studies (Xu et al., 1996a, b and 1998; Zhu et al., 2000; Ruiz-Sánchez et al., 2006), the sperm:oocyte ratio in the present experiment was not corrected for percentage motile sperm after *in vitro* capacitation, thus placing the semen in a context more representative of the *in vivo* situation and avoiding any compensatory effects of sperm numbers used (Saacke et al., 2000; Watson and Behan, 2002). A third factor that could contribute to the low penetration rates obtained in this

experiment is the ejaculate fraction used for IVF evaluation. In previous studies, the sperm peak fraction was used (Xu et al., 1996a, b and 1998; Zhu et al., 2000; Ruiz-Sánchez et al., 2006), as opposed to the complete ejaculate (including subsequent sperm-rich fractions and sperm-free fraction of the ejaculate) in the present study. As early studies demonstrated, when the sperm peak fraction is incubated with seminal plasma from the sperm free fraction, oocyte penetration rate *in vitro* was reduced (Zhu et al., 2000). Furthermore, Rodríguez-Martínez et al. (2005) demonstrated that the sperm from the sperm peak fraction has better characteristics (sperm membrane integrity, % of live cells, etc.) than sperm from the complete ejaculate. Therefore, the lower penetration rates obtained in this experiment could also be attributed to effects from the seminal plasma included in the complete ejaculate and the different sperm population used. Despite all these potential sources of variation among the different studies, the IVF assay used here was able to detect differences between boars that did not differ in conventional evaluated semen characteristics. Nevertheless, a better understanding of the effects of sperm and seminal plasma interactions would allow for further standardization of the ejaculate fraction to be used in obtaining the most reliable prediction of boar fertility.

In summary, the results of this study substantiate the suggestion that the motility of extended semen at Days 7 and 10 of storage offers a practical and inexpensive approach to identify less fertile boars. This approach shows significant potential for commercial use and could have a considerable economic impact by allowing the use of lower sperm doses for AI from the most fertile and genetically high-indexed boars, without losing productivity. Opportunities clearly exist to develop timely and cost-effective procedures for assessing boar fertility in commercial boar studs and further evaluation of these procedures in a commercial setting are warranted.

Table 5-1. Routine semen evaluation results from boars evaluated during the selection period. Boars were considered candidates for this experiment only if their raw semen contained more than 80 % normal sperm with more than 80 % motility. Data are tabulated on the basis of motility group selection, with High (H) and Low (L) motility groups selected for the IVF study on the basis of > or < 60% motility at Day 10.

Boar	Motility Group Selection	Normal Morphology (%)	Raw semen Motility (%)	Motility Day 0 (%)	Motility Day 3 (%)	Motility Day 7 (%)	Motility Day 10 (%)
G	H	97	84	85	80	78	67
I	H	93	83	81	77	73	65
M**	H	92	84	83	82	67	63
L**	H	96	85	84	80	80	63
E	L	95	85	85	78	68	53
D	L	93	81	75	72	62	53
J	L	91	85	84	82	68	48
F	L	94	81	78	77	52	43
C	L	83	80	78	75	65	43
B	NS	85	84	80	83	73	72
H	NS	95	83	82	78	73	65
A	NS	94	82	82	77	67	57
N	NS	93	84	84	73	63	55
O	NS	92	85	82	73	73	55
K	NS	94	84	83	78	73	50

NS: not-selected; **Boars removed from the trial due to logistical problems for IVM/IVF.

Table 5-2. Semen characteristics from the High (n=2) and Low (n=5) motility groups collected: A) During the selection period; B) during the subsequent IVF evaluation period.

A)

Motility Group	Total Sperm per ejaculate (x 10^9)	Normal Morphology (%)	Raw Semen Motility (%)	Stored Extended Semen: Motility Day 0 (%)	Motility Day 3 (%)	Motility Day 7 (%)	Motility Day 10 (%)
HIGH	39.09±8.15	95.2±1.4	83.5±1.5	83±2.8	78.5±2.4	75.5±4.3[a]	66.0±3.2[a]
LOW	43.34±6.17	91.6±0.9	82.4±1.0	80±1.8	76.8±1.5	63.0±2.7[b]	48.0±2.0[b]

Values are least square means (LSM) ± standard errors of LSM.
a-b: LSM with different superscripts within each column were different (P <0.05).

B)

Motility Group	Total Sperm per ejaculate (x 10^9)	Normal Morphology (%)	Raw Semen Motility (%)	Stored Extended Semen: Motility Day 0 (%)	Motility Day 3 (%)	Motility Day 7 (%)	Motility Day 10 (%)
HIGH	66.66±3.7	95.5±1.9	82.5±1.2	51.7±5.9	55.0±17.4	44.2±16.9	45.8±16.1
LOW	72.66±2.3	93.0±1.2	84.0±0.8	62.0±3.8	46.5±11.0	34.3±10.7	25.6±10.2

Values are least square means (LSM) ± standard errors of LSM.

Table 5-3. Penetration rate values for the three different sperm:oocyte ratios used, represented by High (n=2) and Low (n=5) motility groups. IVFTime 1, 2 and 3 represent three consecutive *in vitro* semen evaluations conducted at weekly intervals.

	Sperm:Oocyte Ratio					
	25 x 10^3		**50 x 10^3**		**75 x 10^3**	
	Motility Group		**Motility Group**		**Motility Group**	
IVF Time	**High**	**Low**	**High**	**Low**	**High**	**Low**
1	$62.2\pm8.7^{x\,a}$	$16.8\pm5.5^{x\,b}$	$68.80\pm8.7^{x\,a}$	$16.1\pm5.5^{x\,b}$	$74.07\pm8.7^{x\,a}$	$27.9\pm5.5^{x\,b}$
2	$36.7\pm8.7^{y\,a}$	$25.0\pm5.5^{y\,a}$	$50.90\pm8.7^{x\,a}$	$31.4\pm5.5^{x\,a}$	$64.74\pm8.7^{x\,a}$	$45.1\pm5.5^{x\,b}$
3	$50.8\pm8.7^{x\,a}$	$20.3\pm5.5^{x\,a}$	$59.92\pm8.7^{x\,a}$	$28.2\pm5.5^{x\,b}$	$60.29\pm8.7^{x\,a}$	$32.1\pm5.5^{x\,b}$
	$P=0.018^{*}$		$P=0.103^{*}$		$P=0.121^{*}$	

P* Values for the interaction of IVFtime by motility group within sperm:oocyte ratio.
Values in the table are least square means (LSM) ± standard errors of LSM
Superscripts a,b indicate differences between motility groups within each sperm:oocyte ratio ($P < 0.05$).
Superscripts x,y indicate differences between IVFtimes, but within motility group and sperm:oocyte ratio ($P < 0.05$).

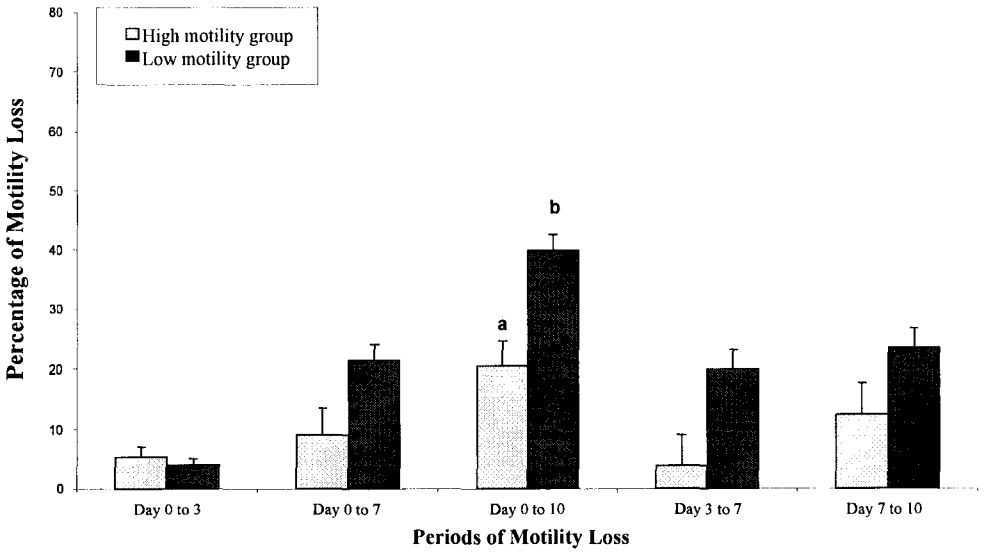
Table 5-4. IVM/IVF results from High (n=2) and Low (n=5) motility groups during the evaluation period using data from the 75 x 10^3 sperm:oocyte ratio evaluation.

In vitro fertilization characteristics	Motility Group	
	High	**Low**
Oocyte penetration rate (%)	66.00 ± 3.50 [a]	35.00 ± 2.20 [b]
Number of sperm penetrated	0.86 ± 0.06[a]	0.37 ± 0.04[b]
Monospermy rate (%)	78.40 ± 4.10[a]	94.50 ± 2.60[b]
Polyspermy rate (%)	16.00 ± 3.00[a]	1.70 ± 2.00[b]
Number of MPN	0.55 ± 0.04[a]	0.22 ± 0.03[b]
MPN-f (%)	49.40 ± 3.50[a]	21.30 ± 2.20[b]
Potential embryo production rate (%)	36.90 ± 2.70[a]	19.70 ± 1.70[b]

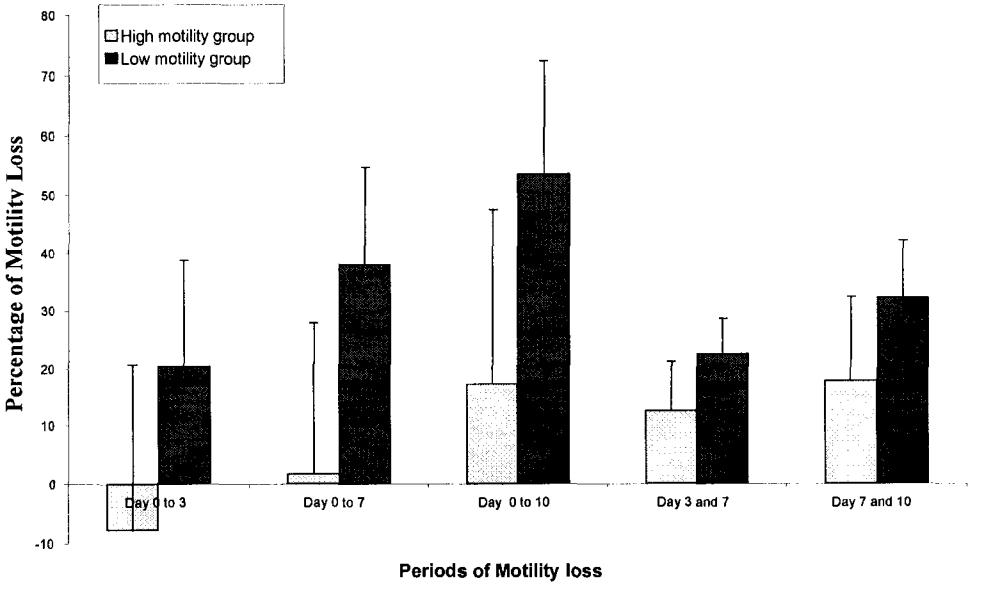
Number of MPN: average number of male pronuclei per penetrated oocyte.
MPN-f: percentage of penetrated oocytes with at least one male pronucleus.
[a-b]: Means with different superscripts within each row were different (P <0.05).
Values in the table are least square means (LSM) ± standard errors of LSM

Figure 5-1. Sperm motility loss at different days of storage of ejaculates from the High (n=2) and Low (n=5) motility groups collected: A) During selection period; B) during subsequent IVF evaluation periods. LS means with different superscripts differ within period ($P < 0.05$). Error bars denote standard error of the LS means.

A)



B)



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CHAPTER SIX

GENERAL DISCUSSION

In the context of animal production, a single male has a more significant impact on farm efficiency and productivity than an individual female, and this impact increases with the use of artificial insemination (AI). As explained in Chapter 1, the use of AI in the swine industry has increased tremendously over the last decade (Burke, 2000; Weitze, 2000) in order to improve boar productivity and accelerate genetic improvement of the herd. This has augmented the demand for good quality processed semen. Unfortunately, there is a lack of good indicators of semen quality and predictors of boar fertility (Flowers, 1997; Rodríguez-Martínez 2003). Therefore, several studies have been conducted to develop practical laboratory tests for the evaluation of semen quality and male fertility potential (Ivannova and Mollova, 1993; Flowers, 1997; Gadea et al., 1998; Juonala et al., 1998; Berger and Parker, 1989; Xu et al., 1998; Gadea and Matas, 2000; Zhu et al., 2000). However, results from only a few of them have been correlated with fertility *in vivo* and/or *in vitro* (Berger and Parker, 1989; Flowers, 1997; Xu et al., 1998 Gadea and Matas, 1999; Tardif et al., 1999; Popwell and Flowers, 2004). Unfortunately, some controversial results have been obtained with these studies, which are perhaps attributable to differences in methodology (range of fertility and/or semen quality evaluated, number of sperm per AI dose, time of insemination time, test methodology, and others). Thus, it is imperative to develop a reliable and practical laboratory test, or tests, to screen for male fertility potential in order to have the ability to differentiate relative fertility amongst boars. This would increase the efficient use of the superior sires and have an enormous economic impact at all the production levels of the swine industry.

Currently, in commercial AI centers, the assessment of boar fertility generally includes a limited evaluation of sperm characteristics and functions (sperm concentration, morphology, viability, and motility) (Gadea, 2005) which are generally

only useful in detecting male infertility (Flowers, 1997; Xu et al.,1998). However, due to the complexity of the fertilization process, once estimates of sperm motility and normal morphology in fresh semen exceed 60 %, these characteristics are not useful predictors of fertility when standard commercial AI doses are used (2 billion or more sperm per AI dose) (Flowers, 1995; Berger and Parker 1989; Flowers, 1997; Flowers and Tuner, 1997; Xu et al., 1998; Popwell and Flowers, 2004). However, the reproductive efficiency of these boars may still be substantially different (Flowers, 1997; Tardif et al., 1999; Popwell and Flowers, 2004), particularly when lower sperm AI doses (1.5 to 0.3 billion sperm per dose) are used (Tardif et al., 1999; Watson and Behan, 2002; Ruiz-Sánchez et al., 2006). Therefore, the general objective of the studies presented here was to evaluate the predictive value of different *in vitro* tests, as well as specific seminal plasma proteins for determining relative boar fertility by using healthy boars with ejaculate quality that otherwise met normal industry standards (>70 % motility and <30% abnormal sperm).

Based on previous studies in our laboratory (Xu et al., 1998), the predictive value of IVM-IVF and routine semen evaluation characteristics were evaluated for assessing boar fertility potential as described in Chapter 3. The IVM-IVF technique assesses the spermatozoa's potential to complete several biochemical and biophysical processes, including capacitation, the acrosome reaction, sperm-zona binding, sperm-oocyte binding and penetration of the oocyte and sperm decondensation, which are all required for successful fertilization. Although just 12 to 17 % of the variation of fertility *in vivo* was explained by the IVM-IVF results obtained from this initial study, the sperm from the less fertile boars performed less well *in vitro,* and the results suggest that it may be possible to establish critical thresholds (e.g. >50 % oocyte penetration rate) for identifying subfertile boars that would reduce farm productivity.

Furthermore, the limited correlations between *in vivo* and IVF data may be produced by the processes involved in IVF methodology. Currently, existing IVM and IVF systems have mainly been optimized for assessing oocyte quality and embryo production potential (Ding and Foxcroft, 1992; Ding et al., 1992; Rath, 1992; Ding et

al., 1994ab; Abeydeera and Day, 1997; Long et al., 1999), as in our first study (Chapter 3). From this we concluded that it was imperative to improve IVM/IVF techniques for sperm evaluation and it would be useful as an *in vitro* tool to understand more about different steps of the fertilization process. Some of the points that have to be considered in optimizing the IVM/IVF technique as a tool for predicting boar fertility are:

1) The use of standardized total sperm numbers per oocyte for IVF, without any adjustments for motility after sperm capacitation *in vitro*. This would place the semen in a more representative context, avoiding any compensatory effects in sperm number used (Saacke et al., 2000; Watson and Behan, 2002).

2) Another approach could be the use of different sperm:oocyte ratios that allow an appropriate ratio to be identified that is less affected by interassay variation, but is sensitive enough to detect the differences between boars, as we established in Chapter 5.

3) Additionally, the use of the same ejaculate fractions for both *in vivo* and *in vitro* fertility evaluations could remove any effect of seminal plasma components. Identification of the ejaculate fraction containing the best sperm subpopulation to test in order to obtain the best predictor of fertility will need further investigation.

4) The use of an internal control in order to identify any problems with the IVM/IVF process that can affect the fertilization results, is also required;

5) Finally, better control of the oocyte quality used in these tests would also contribute to reduced inter-assay variation and increase the repeatability of the test.

As a part of the study described in Chapter 3, the use of low sperm numbers per AI dose identified differences between fertile boars and determined relative boar fertility. This confirmed previous studies (Tardif et al., 1999; Watson and Behan,

2002; Ardón et al., 2003) that established that differences in fertility were revealed when reduced sperm numbers were used for AI. Furthermore, the differences found between the most and least fertile boars (a difference of 15% in farrowing rate and 2 piglets born per litter) and the consistent differences in fertility among boars over the evaluation period, suggests that fertility evaluation of boars immediately after being trained for AI use (approximately 7-8 months old) is feasible. Therefore, less fertile boars can be identified at an early age, by using low numbers of single-boar matings and relatively low sperm numbers for AI. Furthermore, these results provide the basis for establishing a standardized breed-abort protocol, where cyclic gilts from a gilt development program can be used to provide data on pregnancy and potential litter size when aborted at day 30 of gestation. This would allow for the collection of meaningful information on relative boar fertility, without waiting for the bred gilts to farrow, and having little impact on breeding herd productivity.

Another factor that must to be considered during the assessment of boar fertility potential is the seminal plasma composition, and particularly seminal plasma proteins that play a critical role in sperm viability, sperm-oviduct interactions, and sperm-oocyte interactions. As described in Chapter 2, seminal plasma is a complex fluid that affects sperm quality and fertility. In early studies, Flowers (2001) demonstrated that mixing seminal plasma of high fertility ("dominant") boars with the sperm of low fertility ("non-dominant") boars improves the fertility of the sperm used for *in vivo* fertilization. Likewise, Zhu et al. (2000) found differences in oocyte penetration rates when sperm from the sperm peak fraction were pre-incubated with different fractions of seminal plasma from the same ejaculate. These studies confirm the direct effect of seminal plasma components on sperm quality, as determined by differences in fertility *in vitro,* and the importance of including seminal plasma evaluation as a part of the boar fertility evaluation.

Specific seminal plasma proteins have been identified as potential markers of male fertility in the bull (Killian et al., 1993), stallion (Brandon et al., 1999) and the boar (Flowers, 2001). Flowers (2001) working with ejaculates associated with a large

range in fertility (86.7±3.4 to 62.7±4.0 farrowing rates and 11.2±0.3 to 8.6±0.4 average born alive, respectively) was able to identify two seminal plasma proteins (55 kDa, pI 4.5 and 26 kDa, pI 6.2) that were positively associated with *in vitro* and *in vivo* fertility. Following the experiment described in Chapter 3, the association between seminal plasma proteins and observed fertility were examined in Chapter 4. Fertility *in vivo* was negatively correlated ($P < 0.05$) with relative abundance of PSP-I, and with both a 20 kDa, pI 6.0 and 60 kDa, pI 6.5 protein, and positively correlated with a 25 kDa, pI 5.9 protein. Furthermore, a 70 kDa protein in the boars' semen was identified as osteopontin, which has previously been associated with differences in bull (Cancel et al., 1997, 1999) and stallion fertility (Brandon et al., 1999). Collectively, these results confirm that specific seminal plasma proteins are indeed associated with boar fertility, providing the basis for their use as a complementary tool to identify sires with high and low fertility potential.

Flowers (2001) reported that differences in a 55 kDa, pI 4.5 and 26 kDa, pI 6.2 proteins appeared to be associated with differences in fertility *in vivo.* Our data demonstrated that this protein appears to be equivalent to protein 35 identified by 2-D gel analysis. However, no correlations with fertility were found in our study. This is likely due to the use of boars with a narrower range of fertility, thus excluding the more infertile boars that appeared to create most of the variance in both fertility and the amount of the 55 kDA protein in the experimental paradigm reported earlier by Flowers (2001). Furthermore, the fact that these data have yet to be published in the peer-reviewed literature brings the data and/or methodology used into question. Meanwhile, the positive correlations found with fertility and a 25 kDa, pI 5.9 protein, suggest that this protein could be homologous to a fertility associated seminal plasma protein in the boar (Flowers, 2001), bull (Killian et al., 1993) and the stallion (Lipocalin-type prostaglandin D synthase (Gerena et al., 1998; Brandon et al., 1999)). The specific role of this protein is still not confirmed, but Gerena et al. (1998) suggest a role in sperm development and maturation due to its presence on the apical ridge of the sperm acrosome. The identification of this protein in the boar and its role during the fertility process require further investigation.

Differences in the amount of seminal plasma proteins between different fractions of the ejaculate were also confirmed in Chapter 4, where the Sperm-Peak fraction possessed lower total protein concentrations, as well as lower concentrations of specific seminal plasma proteins (PSP-I, AWN-1 and OPN), than the Sperm-Free fraction. This effect could be produced by a combination of factors: 1) seminal plasma origin, 2) sperm quality (sperm ability to absorb seminal plasma proteins), and/or 3) sperm concentration. These factors may, individually or collectively, determine the variation in total protein concentration among the seminal plasma fractions.

Our data in Chapter 4 showed that total seminal plasma protein concentration was negatively correlated with both zona pellucida penetration rate and the number of sperm penetrating the zona. This, along with differences in specific seminal plasma proteins (Spermadhesins and decapacitation factors) between Sperm-Peak and Sperm-Free fractions, could explain the reduction in penetration rates observed by Zhu et al. (2000), when sperm from the Sperm-Peak fraction were pre-incubated with seminal plasma from the Sperm-Free fraction. In a pilot study (Appendix 1, Table A-1), differences in IVF parameters between ejaculate fractions (Sperm-Rich vs. Sperm-Peak) were evaluated. Although most of the characteristics associated with higher fertility were superior for the Sperm-Peak fraction, only average number of sperm penetrating the zona pellucida, and average number of sperm penetrating the oocyte, were found to be significantly increased. In contrast, in an *in vitro* study, the inclusion of seminal plasma in sperm samples sorted by flow-cytometry increased the percentage of uncapacitated, acrosome intact sperm and reduced oocyte penetration rate (Maxwell and Johnson, 1999). Furthermore, recent studies have demonstrated that exposure to PSP-I/PSP-II heterodimers maintains sperm motility, viability, and mitochondrial activity in highly extended ejaculates (Centurion et al., 2003; García et al., 2003; Caballero et al., 2006), as well as in frozen semen (Caballero et al., 2004). Collectively, these results suggest that seminal plasma proteins have a predominantly decapacitating effect, reducing sperm penetration *in vitro*.

The assessment of sperm motility at the time of collection is an indirect measurement of sperm viability that is only useful in estimating the reproductive performance of boar semen that has less than 60 % of motile spermatozoa when commercial doses are used (<2billion sperm per AI dose) (Table 2-5) (Flowers, 1997; Xu et al., 1998). However, Tardif et al. (1999) demonstrated that when using lower numbers of sperm per AI dose (0.3×10^9 sperm per AI dose), the percent of sperm with normal motility was positively correlated with farrowing rate (r=0.783, P=0.01). This suggests that the use of large numbers of sperm per AI dose may mask actual boar fertility potential when relatively fertile boars are compared. Nevertheless, our data showed a significant boar by time interaction for the motility of raw semen, and given the very consistent differences in relative boar fertility *in vivo* over time, this would strongly suggest that this parameter is not suitable as a predictor of boar fertility.

In contrast, sperm motility of extended storage semen has been correlated with *in vitro* fertility estimates (Xu et al., 1996b). However, the correlations with *in vivo* fertility have been controversial (Juonala et al., 1998; Xu et al., 1998; Sutkeviciene et al. 2005; Mezalira et al., 2005). In the study described in Chapter 3, differences in the motility of extended semen at Days 7 and 10 were significantly correlated with *in vivo* fertility. This led to the evaluation of the changes in sperm motility of storage semen as an indicator of relative boar fertility (Chapter 5). Our results demonstrated differences between boars in sperm motility of extended semen at Days 7 and 10 of storage. Furthermore, the ability to identify sub-populations of potentially low or high fertility boars, based on differences in motility of extended semen, was confirmed, and a positive correlation between motility of stored semen and boar fertility was established. This provides the basis for using this practical test as a complementary tool to identify boars with high and low fertility potential, at an early age.

Furthermore, the controversial results found in earlier studies (Xu et al., 1996a; Mezalira et al., 2005) may be an artifact of the motility evaluation methodology. For

this reason, the following important points must be considered when the motility of extended stored semen is used as a predictor of boar fertility:

1) The type of extender (short vs. long term) used to extend and store the sample. Xu et al., (1996a) using short term extender (BL-1), found a significant correlation ($r=0.8989$; $P<0.0001$) between Day 7 motility and penetration rate; however, in a subsequent study the same research group used a long-term extender (Androhep) and no significant correlations between motility at Day 7 and fertility *in vivo* were found (Xu et al., 1998). Therefore, given the inherent ability of long-term extenders to preserve sperm characteristics (Huo et al., 2002; Dubé et al., 2004; Vyt et al., 2004; De Ambrogi et al., 2006), the storage time needed to observe loss of motility may is observed may be longer than with short-term extenders.

2) Another consideration is how the assessment is carried out. Ideally, the same well trained personnel will perform the assessment.

3) Likewise, during sperm motility evaluation, a series of important factors must be considered in order to reduce the chance of under or over estimations for a sample: a) Incubation time and temperature of the semen sample at the time of evaluation (20 to 30 min and 36 to 37°C, respectively); b) pre-warm all the equipment used for the evaluation; c) size of the sample must be standardized according to the size of the cover slip used (9 to 15 μL drop); d) sperm concentration between samples; and e) the use of caffeine; 4) Finally, if more precise methods of measuring sperm motility and motility characteristics such as CASA are going to be used, technical difficulties inherent to these systems (e.g. selection of gel particles instead of sperm shapes, over and/or under estimations) have to be considered in order to increase their repeatability and accuracy.

6.2. GENERAL CONCLUSIONS

In conclusion, the evaluation of IVM/IVF as a predictor of boar fertility demonstrated that this technique is able to discriminate between relatively fertile boars in terms of their true potential productivity under more stringent (lower sperm numbers per dose) or demanding (use of ageing stored semen) conditions of AI use. However, further studies are needed to optimize this technique and increase its sensitivity and repeatability, for a better estimation of fertility. Furthermore, the use of this test in conjunction with other *in vitro* tests will result in the assessment of different aspects of the fertilization process that are not evaluated with the IVM-IVF alone, and may increase measured associations with fertility *in vivo*. Complementary tests and/or techniques to considered include: separation of viable spermatozoa (swim-up test or Percol separation (Grant et al., 1994; Bouvet et al., 2005)); sperm-oviductal binding assay (Petrunkina et at., 2001; Waberski et al., 2005); and the effect of extended stored semen on sperm motility (Ruiz-Sánchez et al., 2005).

The results presented in Chapter 4 provide strong evidence of specific boar seminal plasma proteins that may mediate observed differences in semen quality and boar fertility. The identification of these fertility markers will provide the basis for using them to identify sires with high and low fertility potential, having a considerable economic impact in the swine industry.

On the other hand, the use of lower sperm numbers per AI dose and the implementation of the breed and abort protocol, can provide meaningful information on relative boar fertility without waiting for the bred gilts to farrow, thereby having little impact on breeding herd productivity and making it more suitable for use in fertility trials.

Finally, this research also demonstrates that the evaluation of motility of extended stored semen provides a practical and inexpensive approach to identifying less fertile boars at an early stage. Once identified, measures can be taken (boar

elimination or use of alternative AI techniques), before they have a negative effect on farm productivity. However, evaluation of this technique at a commercial level is required to confirm this relationship and optimize the methodology to meet the requirements of a commercial environment.

6.3. IMPLICATIONS

The ability to differentiate relative fertility amongst boars would have significant economic impact on the swine industry, by eliminating or optimizing the use of less fertile boars. It would also allow for the use of lower sperm AI doses for the most fertile and genetically high-indexed boars, without any loss in productivity.

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APPENDIX

APPENDIX 1: Effects of ejaculate fractions on IVF Parameters

Table A-1. Samples of Sperm-Peak and Sperm-Rich fractions from six boars were evaluated by IVF and ten parameters were used to compare the ejaculate fraction effect.

Sperm characteristic evaluated	Ejaculate Fraction	
	Sperm-Rich	**Sperm-Peak**
Zona Pellucida Penetration rate (%)	85± 4	93±4
Average SZP	6.61±3.6[a]	18.22±3.6[b]
Oocyte Penetration rate (%)	63± 7	76±7
Average SPO	1.98±0.4[a]	3.08±0.4[b]
Polyspermy (%)	48±7	60±7
Monospermy (%)	52±7	40±7
Male Pronuclear formation (%)	73±5	73±5
Potential embryo formation (%)	33±5	24±5

Values in the table are least square means (LSM) ± standard errors (SE) of LSM.
[a-b]: Means with different superscripts within each row were different ($P < 0.05$).
Average SZP: average number of penetrated sperm the zona pellucida.
Average SPO: average number of penetrated sperm per oocyte.

APPENDIX 2: Percentage of Seminal plasma per extended AI dose

Table A- 2. Boar by time effects (P<0.0001) on percent of seminal plasma per extended AI dose from all nine boars for each breeding period (Time) during the evaluation period (6±1 mo).

	Boars								
Time	**B-1**	**B-3**	**G-1**	**G-2**	**Pu-3**	**R-1**	**R-2**	**R-3**	**Y-2**
1	7.1±1.2	8.1±1.2	8.4±1.2	6.1±1.0	9.6±1.4	9.1±1.2^{x}	6.8±1.0	7.2±1.4	5.6±1.0
2	7.0±1.2^{a}	6.6±1.2^{a}	8.2±1.2^{ab}	7.1±1.1^{a}	9.5±1.2^{a}	20.7±1.2^{by}	6.3±1.1^{a}	7.2±1.2^{a}	5.7±1.0^{a}
3	5.8±1.1	5.6±1.0	6.4±1.1	7.8±1.2	8.4±1.0	8.2±1.1^{x}	7.6±1.2	8.9±1.2	5.4±1.2
4	5.6±1.1	6.1±1.0	6.8±1.1	7.8±1.1	7.8±1.1	11.9±1.1^{x}	7.8±1.1	6.8±102	5.0±1.1
5	6.5±1.1	6.4±1.0	8.5±1.1	9.0±1.1	7.1±1.0	10.9±1.1^{x}	7.2±1.1	6.5±1.0	6.0±1.1
6	7.2±1.1	6.1±1.1	6.3±1.1	8.9±1.1	7.7±1.1	9.9±1.2^{x}	6.9±1.1	6.3±1.1	6.5±1.1
7	5.3±0.9	6.6±1.2	4.9±0.9	8.6±1.2	7.0±1.2	6.1±1.0^{x}	8.4±1.2	6.9±1.2	6.4±1.2

Values in the table are least square means (LSM) ± standard errors (SE) of LSM.
$^{a-b}$: LSM±SE with different superscripts within row each were different (P<0.05).
$^{x-y}$: LSM ± SE with different superscripts within each column were significant different (P<0.05).

APPENDIX 3. Optimum centrifugation speed for preparation of seminal plasma samples for protein analysis.

Table A-3: Total protein concentration differences after using different centrifugation speeds for seminal plasma recovery from ejaculates of three different boars. Treatments: A) spin at 1,000g for 30 min, harvest and freeze the seminal plasma, and then thaw and re-spin the plasma at 10,000g for 15 min before analysis; B)spin at 10,000g for 15 min and then harvest and freeze the seminal plasma; C) spin at 1,000g for 30 min, harvest the seminal plasma, and the spin again at 10,000g before harvesting the plasma a second time before freezing the sample; D) spin at 1,000g for 30 min and then harvest and freeze the sample.

Boar	**Treatments**			
	A	**B**	**C**	**D**
1	12.32	13.28	13.16	13.42
2	26.29	26.98	26.03	27.63
3	26.73	29.02	28.56	29.83
SEM	21.78±6.92	23.10±6.92	22.58±6.92	23.63±6.92

APPENDIX 4: Comparison of sperm concentration techniques

Figure A- 1. Linear Regression lines and 95% prediction intervals using values of two evaluators of the comparison between Manual Haemocytometer vs. Colorimeter. Sperm concentrations obtained with the colorimeter was highly correlated with the "Gold Standard" haemocytometer values and showed low variation (a coefficient of variance of 6.53%), a high correlation ($R^2 = 0.91$, $P<0.05$) and no absolute differences ($P<0.05$) between technicians.