LOCALIZATION ON SPERM, QUANTIFICATION AND MOLECULAR FEATURES OF TWO SEMINAL PROTEINS

by

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DEDICATION

To my grandmother, mother and brother, I dedicate this accomplishment to you.

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ABSTRACT

Objective markers to identify higher fertility individuals are needed to maximize livestock breeding success. Two heparin-binding proteins, which are reflective of fertility in bulls, have been biochemically identified as fertility-associated antigen (FAA) and tissue inhibitor of metalloproteinases-2 (TIMP-2). These four studies were designed to examine the importance of those proteins in relation to reproduction in bulls and other livestock species.

In the first study, indirect immuno-fluorescent microscopy was performed to localize FAA and TIMP-2 to livestock sperm. FAA was localized on spermatozoal acrosomes of bulls and rams, but no cross-reactivity was observed for stallions. TIMP-2 labeling was observed on acrosomes and posterior heads, which was species dependent. Localization patterns for FAA and TIMP-2 were further investigated during heparin-induced capacitation and acrosome reactions of bovine sperm.

In study two, an enzyme-linked immunosorbent assay (ELISA) was developed to determine concentrations of FAA in bovine seminal plasma (SP). A commercially available TIMP-2 ELISA was utilized to quantify TIMP-2. Respective mean concentrations of FAA and TIMP-2 in SP were 6.66±1.487 ug/ml and 1.18±0.045 mg/ml. Concentrations of FAA in SP did not correspond to bull fertility potential, however, older bulls with higher concentrations of TIMP-2 in SP sired more calves.

The third study evaluated utility of an amplified fragment length polymorphism with bovine TIMP-2 gene specific primers to amplify a 700 bp genomic DNA (gDNA) product from sperm. From 53 bulls screened, 22.6% were negative for the 700 bp

amplicon. There was a three-fold likelihood for 700 bp negative bulls to not sire a calf compared to 700 bp positive bulls. The product was cloned and sequenced, but no homology to TIMP-2 was detected. Therefore, the product represented novel bovine gDNA sequence.

The fourth study identified an equine homologue to the bovine FAA gene. Immuno-based diagnostics had not detected FAA in stallion semen. The equine DNA homologue was 88.5% identical in nucleotide and 86% in amino acid sequences to bovine FAA. Subtle differences in the amino acid sequence are likely responsible for the inability to detect FAA in stallion semen with FAA antibodies to bovine FAA.

CHAPTER ONE

I. PRESENTATION OF THE PROBLEM

Reproduction has been proposed to be five to 10 times more important, as a trait to commercial beef operations, than any other measurable trait related to performance (Trenkle and Willham, 1977). Therefore, efficiency of reproduction is the most important factor affecting overall efficiency of production in most domestic animal species (Bradford et al., 1991). In beef herds, a calving rate of 90% is most desirable, however, the average across the United States is estimated to be 70 to 80%. Data available from the United States Department of Agriculture (National Animal Health Monitoring System) indicate that a reduction in cow/calf reproductive efficiency by one percent would result in approximately 4.5 million dollars in economic loss across the United States. Failed reproduction accounts for over two billion dollars lost each year.

Those values are alarming and further indicate the importance of selecting for fertility when choosing replacement males and females in livestock breeding operations. The difficulty resides in the fact that no objective markers are available for fertility potential to identify the most fertile males and females. In bulls, exams are performed by veterinarians that "qualify" a bull as a potential satisfactory breeder or not. Those tests are known as breeding soundness exams (BSE) and consist of assessing scrotal size and semen parameters (sperm motility, morphology and concentration) along with the general health of the animal. The fertility of range bulls was increased successfully when bulls were subjected to a BSE (Chenoweth et al., 1992). However, bulls that pass the BSE may still vary in actual fertility potential (Bellin et al., 1994; 1996; and 1998). Recently,

a commercially available test for a fertility-related protein in bull semen has entered the marketplace that is to be used in conjunction with the BSE to aid producers in selecting bulls that are more fertile (McCauley et al, 2004).

Although bench-side progress is evolving into the field to identify bulls which are higher in fertility, that is not the case for other livestock species. As more seminal constituents continue to be identified and their molecular mechanisms by which they regulate male reproduction become unraveled, an emphasis needs to be placed on their relationship to fertility. The development of a panel of biochemical markers for fertility that could be used across species would have a tremendous impact on the livestock industry.

The remainder of this chapter provides an overview of the literature in regards to bioactive molecules that interact with sperm, influence sperm function and are related to male fertility.

II. REVIEW OF THE LITERATURE

For successful fertilization to occur, sperm must undergo capacitation. The earliest descriptions of capacitation were by Chang (1951) and Austin (1952). It is defined as the final maturational step that sperm must undergo in preparation for fertilization (Austin, 1952). Sperm undergo those capacitative changes during transport in the female reproductive tract in preparation for the final release of acrosomal contents, termed the acrosome reaction, which allows for penetration and fertilization of ova (Yanagimachi, 1994). To date, there remains no clear recognizable marker for capacitation and the events which precede this poorly defined process of sperm maturation. However, several intracellular changes are known to occur prior to the acrosome reaction (the endpoint of capacitation). Those include an increase in membrane fluidity, cholesterol efflux, alkalinization of internal pH (pH_i), elevated intracellular Ca^{2+} and cAMP concentrations and protein phosphorylation.

The identification of physiological inducers of capacitation from the female reproductive tract has led to the development of *in vitro* culture systems that allow for the evaluation of sperm fertilizing capabilities.

A. *In vitro* Capacitation of Sperm

Glycosaminoglycans (GAGs) or mucopolysaccharides are linear unbranched polysaccharides. GAGs are synthesized attached to a protein backbone, which form a proteoglycan. Heparin, a common anticoagulant in blood, is the most commonly known glycosaminoglycan. Heparin most commonly consists of alternating residues of D-iduronate-2-sulfate and *N*-sulfo-D- glucosamine-6-sulfate, and it contains approximately

2.5 sulfides per disaccharide unit (Voet and Voet, 1995). GAGs have been implicated as some of the compounds in the female reproductive tract secretions that capacitate spermatozoa (Lee and Ax, 1983; Lenz et al., 1983; Lee et al., 1986). Heparin is the most potent GAG to promote capacitation, and its degree and positions of sulfation are essential for its capacitating biological activity (Miller and Ax, 1989).

Glycosaminoglycans have been chemically identified in every region of the female reproductive tract, including follicular fluid, in cattle (Lee and Ax, 1984), ewes (Lee et al., 1986), mares (Varner et al., 1991), and humans (Kitamura et al., 1980). Under the influence of estrogen, more highly-sulfated GAGs are present in the tract secretions compared to secretions under progestational dominance (Lee and Ax, 1984; Lee et al., 1986). Therefore, during the normal changes associated with the estrous cycle, more potent GAGs in terms of facilitating capacitation are present at times when spermatozoa would be transported to the site of fertilization.

Heparin facilitates sperm to undergo capacitation *in vitro* in several livestock species including bull (Parrish et al., 1988; 1989), ram (Slavik et al., 1990; Slavik and Fulka, 1991) and stallion (Farlin et al., 1993; Varner et al., 1993). Although bull and ram sperm respond similarly to a given dose of heparin (10 ug/ml), the dose of heparin that effectively capacitated stallion sperm ranged from 10 to 100 ug/ml (Farlin et al., 1993; Varner et al., 1993; Christensen et al., 1996; Merkies and Buhr 1998). The measured response to heparin by stallion sperm indicates unique differences between species exist, and although unknown, it may be reflective of fertility potential. The ability of bull sperm to respond to a given dose of heparin has been correlated to bull fertility. Marks

and Ax (1985) identified that higher fertility bulls produced sperm that are more susceptible to the capacitating effects of a given dose of heparin compared to lower fertility bulls. Moreover, higher fertility bulls also produced sperm, which displayed a higher affinity to binding labeled heparin compared to lower fertility bulls (Marks and Ax, 1985). The relationship between fertility and the ability of sperm to bind heparin in other species is not well described.

The beneficial effects of using heparin to capacitate sperm are well established. More recently, the use of heparin and other carbohydrates and glycoproteins has been investigated to improve the success of bovine fertilization and early embryonic development in vitro (Li et al., 2004; Tanghe et al., 2004). Heparin-containing media, when used post-fertilization, had a beneficial effect on pronuclear formation and cleavage rate in developing bovine embryos (Li et al., 2004). The methods by which heparin influences post-fertilization development is unknown, but it is likely through similar intra-cellular signaling pathways that affect sperm, which ultimately elevate intracellular Ca^{2+} concentrations.

B. Intracellular Signaling Required for the Acrosome Reaction

Once mammalian spermatozoa undergo capacitation, they acquire the ability to acrosome react. The acrosome reaction is described as an exocytotic, calcium-dependent event (Dan, 1954; Yanagimachi and Usui, 1974) and the endpoint of capacitation (Yanagimachi, 1994). As previously discussed, heparin has been shown to bind to bovine sperm. It also exerts effects on the calcium uptake by sperm and the intracellular pH (Handrow et al., 1989; Parrish et al., 1989). The increase in intracellular pH has been

associated with changes in Na^+, K^+ and HCO_3^- ions. It is proposed that Ca^{2+} and HCO_3^- are required for the activation of sperm adenylyl cyclase (Visconti et al., 1995). Adenylyl cyclase would result in cAMP production and the activation of protein kinase A (PKA), which would phosphorylate certain proteins.

Phosphorylation plays a major role in the intracellular signaling cascade stimulated during capacitation (reviewed by Visconti et al., 2002). Induction of capacitation of mammalian sperm stimulated a cAMP-dependent increase in tyrosine phosphorylation (Galantino-Homer et al., 1997; Ficarro et al., 2003). In bulls, the appearance of tyrosine phosphorylated proteins correlated with the time course of capacitation induced by heparin, and was dependent on the concentration of heparin. Tyrosine phosphorylation of ram sperm membrane proteins, in relation to capacitation, have also been described (Perez-Pe et al., 2002). Recently, Galantino-Homer et al. (2004) using heparin, demonstrated that intracellular alkalinization is a key process which allow bovine sperm to undergo the acrosome reaction and that protein tyrosine phosphorylation correlated with capacitation of bovine sperm. However, alkalinization and protein phosphorylation were also achieved by increasing extracellular pH (pH_o) in the absence of heparin. That finding was validated by use of a fusogenic reagent (lysophosphatidylcholine) to induce sperm to acrosome react following a 4-h culture. The method by which heparin promotes capacitation in the bovine, as well as other species, is largely unresolved, but interactions of GAGs with proteins on sperm offer a useful means to study capacitation *in vitro* as well as *in vivo*.

C. Heparin-binding Proteins in Semen

Numerous seminally derived proteins have been identified in semen that bind heparin. Chandonnet et al. (1990) isolated a series of bovine seminal proteins (BSPs) that are secreted by the seminal vesicular glands. Those proteins were termed BSP-A1, BSP-A2 and BSP-A3 with a molecular mass of 15-17 kDa, respectively, and a larger 28-30 kDa protein identified as BSP-30. BSP-A1 and BSP-A2 were previously identified as the PDC-109 protein (Esch et al., 1983) and later determined to be structurally similar by Calvete et al. (1994a). Biochemically, the BSPs are acidic in nature with isoelectric points (pI) ranging from 3.6-5.2 and all except for BSP-A3 are glycosylated (Manjunath et al., 1988; and Manjunath and Therien, 2002). Homologues of the BSP proteins have been identified in hamster, rat, mouse, boar, human (Leblond et al., 1993; Calvete et al., 1997), stallion (Calvete et al., 1994b; 1995; 1997; and Menard et al., 2003), goat (Villemure et al., 2003) and buffalo (Boisvert et al., 2004). Quantification studies have indicated the BSP proteins represent approximately 40 to 57% (31-46 mg/ml) of the total protein in bovine seminal plasma and 4 to 6% of the total protein (1.8-2.6 mg/ml) in sperm (Nauc and Manjunath et al., 2000). The variation in measured BSP concentrations was high from bull to bull and also from ejaculate to ejaculate within bull. In addition, there was no relationship established to fertility of Holstein bulls.

Immunofluorescence studies revealed intense labeling of anti-BSP antibodies over the acrosome, post-acrosome and midpiece of ejaculated bovine sperm, but labeling was not detected on epididymal sperm (Manjunath et al., 1994). In addition to binding heparin, the BSPs also bind to choline phospholipids (Desnoyers and Manjunath, 1992)

present in the lipid bilayer of bovine sperm. Bovine sperm are rich in multiple forms of choline phospholipids (Parks et al., 1987) so those lipids may provide an attachment site for seminal derived BSPs.

As discussed previously, heparin and heparin-like molecules effectively capacitate bovine sperm. Likewise, high-density lipoproteins (HDL) have been shown to capacitate bull sperm (Therien et al., 1997) and HDLs bind to the BSPs (Manjunath et al., 1988; 1989). The method by which BSPs modulate capacitation by interacting with those molecules is apparently different. Tyrosine phosphorylation levels significantly increased when sperm were cultured in the presence of heparin, while phosphorylation levels did not change when HDL was used to capacitate bull sperm (Lane et al., 1999). The mechanism by which BSPs regulate capacitation induced by HDL is proposed in a review by Manjunath and Therien (2002), but the inability to account for the lack of protein phosphorylation was not addressed. The BSP's interaction with membrane-associated lipids and modifications of the lipid bilayer (i.e., cholesterol efflux) may be where the important functions of those heparin-binding proteins reside.

Miller et al., (1990) also isolated a series of heparin-binding proteins (HBPs) from bovine seminal plasma using heparin-affinity chromatography. The molecular masses of those proteins were 15-17 kDa, 24 kDa and 31 kDa. Purified fractions of those proteins were added to epididymal sperm, and they conveyed the capacitating effects of heparin (Miller et al., 1990). Furthermore fractions which contained the 24 and 31 kDa proteins possessed greatest activity/unit of protein to induce acrosomal exocytosis (Miller et al., 1990). Also in 1990, Nass and coworkers established that those proteins were produced

by the bulbourethral, prostate and seminal vesicular glands by rats and bulls. Interestingly, concentration of HBPs were significantly reduced after rats were castrated, and pre-castration concentrations of HBPs were restored by testosterone supplementation. Although testosterone appears necessary for HBP production, the exact concentrations of circulating androgens needed remain to be determined.

To date, the 15-17 kDa proteins discussed above have not been biochemically identified. However, those proteins may be identical or homologues to the BSP-A1/BSP-A3 or PDC-109 proteins (Esch et al., 1983; Chandonnet et al. 1990). The 24 and 31 kDa peptides originally identified in bovine seminal plasma (Miller et al., 1990) have been purified and biochemically identified. The 31 kDa protein "coined" fertility-associated antigen (FAA; Bellin et al., 1998) was characterized by N-terminal sequencing and Lys-C digestions of FAA and sequencing of those internal fragments (McCauley et al., 1999). Amino terminal and two digested fragments were reported as being 73%, 85% and 92%, respectively, to human deoxyribonuclease (DNase) I-like protein. In addition, FAA was non-glycosylated and basic with an isoelectric point of ~7.5-8.0 (McCauley et al., 1999). No similarities were shared between FAA and the 30-kDa amino acid sequence of BSP-30 (Salois et al., 1999). Thus, FAA is a unique protein and the first report of a novel DNase I-like isoform in bovine semen. FAA accounts for approximately 0.5% of total protein in seminal fluid from a vasectomized bull (McCauley et al., 1999).

The 24 kDa HBP, was also purified and characterized from bovine seminal fluid, and its amino terminus shared 90% identity (McCauley et al., 2001) to a previously identified tissue inhibitor of metalloproteinases-type 2 (TIMP-2) isolated from bovine

aortic endothelial cells (DeClerck et al., 1989). TIMP-2 mRNA expression was also identified in bovine tissue harvested from the prostate, bulbourethral and seminal vesicle gland (McCauley et al., 2001). TIMP-2 had previously been identified in bovine seminal plasma as a major seminal plasma protein (Calvete et al., 1996), but the site of origin and potential relationship to bull fertility was not known. Liberda et al. (2001) also confirmed the presence of TIMP-2 in bovine seminal fluid. TIMP-2 has been detected in the male reproductive tract or in semen from humans (Shimokawa et al., 2003; Baumgart et al., 2002a and 2002b), rats (Longin et al., 2001; Siu and Cheng, 2004), rams and stallions (Metayer et al., 2002).

DNase and metalloproteinase inhibitor (TIMP) activity have been proposed as regulators of male reproduction. DNase I activity has been confirmed in human (Singer et al., 1983; Yasuda et al., 1993), bull (Tanigawa et al., 1983), mouse (Carballada and Esponda, 2001) and rooster (Sato et al., 2003) semen. In the rat, DNase I is proposed to modulate germ cell numbers by apoptosis (Stephan et al., 1996) by induction of endogenous DNA degradation as described by Peitsch et al. (1993). The role of DNases (I or I-like) in ejaculated semen remains to be determined. However, the DNase I-like isoforms possess important biological motifs. They include calcium-binding and DNA-binding domains along with sites for signal peptide cleavage (Rodriguez et al., 1997). The sequestering of intracellular calcium by sperm is one, clear prerequisite to capacitation and the subsequent acrosome reaction (reviewed by Breitbart, 2002a; 2002b). Moreover, the gene that encodes for DNase I predicts a glycosylated protein, while DNase I-like genes encode for non-glycosylated proteins. That agrees with the

findings of McCauley et al. (1999) and suggests that FAA may be an isoform of the DNase-I like family. To date, there has been no report in the literature of FAA having DNase activity, while all other I-like isoforms possess this biological activity.

Tissue inhibitors of metalloproteinases (TIMPs) are members of the matrix metalloproteinases (MMPs), a large family of proteolytic enzymes (reviewed by Sternlicht and Werb, 2001). TIMPs inhibit the catalytic activity of MMPs and thereby regulate degradation of extracellular matrix (ECM) in both normal and disease states (Gomez et al., 1997; Nagase et al., 1999). The MMPs have been considered as mediators of reproductive function (Hulboy et al., 1997), which includes ovulation, implantation, parturition, involution, prostate and testicular function. Research in mice, however, would indicate that fertility, growth and development did not require TIMP-2 when an inactivating mutation was introduced into the TIMP-2 gene (Caterina et al., 2000). In addition to regulation by TIMPs, several reproductive hormones and numerous growth factors regulate MMPs as well (reviewed by Hulboy et al., 1997). TIMP-2 is known to regulate membrane-type MMPs by inhibiting the cleavage or conversion of pro-MMP to the active MMP zymogen form (Brew et al., 2000). MMPs have been localized to the acrosome and midpiece of normal and abnormal human sperm (Buchman-Shaked et al., 2002). However, localization of TIMP-2 on sperm has not previously been established. The exact role of the TIMPs and MMPs on sperm or in semen is not known. TIMPs may serve as protease inhibitors in mammalian semen and/or protect sperm from enzymatic digestion by the MMPs.

The well documented presence and abundance of TIMP-2 in bovine semen and its ability to bind heparin (Calvete et al., 1996; Liberda et al., 2001; and McCauley et al., 2001) suggests that it has an important relationship to reproduction in bulls. Future studies are needed that evaluate the interaction of TIMP-2, MMPs and heparin, since heparin appears to act as an extracellular attachment site for specific MMPs (Yu and Woessner, 2000).

D. Protein Markers in Semen for Bull Fertility

There are four proteins that have been biochemically identified in bovine semen that are reflective of fertility potential in bulls. They are osteopontin (OPN), lipocalin-type prostaglandin D synthase (PGD), fertility-associated antigen (FAA) and tissue inhibitor of metalloproteinases-2 (TIMP-2). Their discovery, relationship to fertility and potential function(s) of those proteins are discussed in the following two sections.

1. Osteopontin and Lipocalin-type Prostaglandin D Synthase

Killian et al. (1993) identified four seminal plasma proteins in Holstein bull semen that were associated with bull fertility. They consisted of a 55 kDa (pI 4.1), 26 kDa (pI 6.2), 16 kDa (pI 6.7) and 16 kDa (pI 4.1) protein utilizing two-dimensional (2D) SDS-PAGE separation techniques. By analyses of protein densities, the first two, later characterized as osteopontin (55 kDa; Cancel et al., 1997) and lipocalin-type prostaglandin D (PGD) synthase (26 kDa; Gerena et al., 1998) were more prominent in higher fertility bulls. The two 16 kDa proteins, which are uncharacterized, were prevalent in low fertility bulls. Based on relative protein density values, OPN was positively correlated (r=0.48) with fertility (Cancel et al., 1997). When bulls are

categorized as above average or below average fertility, lipocalin-type PGD synthase is 3.5 times more prevalent in the above average group of bulls (Killian et al., 1993; Gerena et al., 1998; 2000). The work of Killian and others has led to a multiple regression model (R=0.89) to predict the fertility of bulls based on these four fertility-associated proteins (for equation see Killian et al., 1993).

Currently, the cellular roles of OPN and lipocalin-type PGD synthase and their influence on bull fertility remain to be elucidated. However, OPN plays an integral part in a number of signal transduction pathways (for review see Denhardt et al., 2001) including defense mechanisms and inflammatory conditions. OPN is widely expressed and distributed throughout a number of different tissues including skeletal bone, gastrointestinal tract, mammary glands, lungs, salivary glands, sweat glands and the reproductive tract of humans (Brown et al., 1992). However, in relationship to the reproductive tract of bulls, OPN has only immunohistochemically been shown to be present on the epithelial surface of the ampulla and seminal vesicular glands (Cancel et al., 1999). Its primary contributions to semen are believed to originate from those specific accessory sex glands, as OPN was not localized to tissue from the testis, epididymis, prostate or bulbourethral gland (Cancel et al., 1999). In addition, OPN may serve as a functional cell attachment protein to provide transitory stabilization of the sperm plasma membrane prior to fertilization.

Localization studies have identified lipocalin-type PGD synthase to be present within elongating spermatids, Sertoli cells, rete testis and efferent duct epithelial cells along with epididymal epithelial cells (Gerena et al., 2000). PGD synthase is responsible

for the synthesis of PGD_2, a endogenous sleep-inducing factor which also acts to control other homeostatic systems including body temperature, LH release and odor modulation (Hayaishi et al., 1993). In addition, fluorescent microscopy revealed the presence of lipocalin-type PGD synthase on the apical ridge of the acrosome on ejaculated bovine sperm (Gerena et al., 2000). This evidence would suggest lipocalin-type PGD synthase may play an important role in the spermatogenic cycle, as well as maturation of spermatozoa during storage in the epididymis. The possibility too exists for it to serve as a lipophilic carrier within the reproductive tract, thus acting as a transmembrane carrier protein for maintenance of the blood-testis barrier or blood-epithelial barriers. It is also important to note other proteins like androgen-binding protein for sequestering testosterone and retinol-binding protein for retinoic acid are members of the lipocalin-transport family (Voet and Voet, 1995). These lipocalins are capable of binding hydrophobic molecules and transporting them across tissue barriers. Lipocalin-type PGD synthase's multifaceted sites of localization and activity lend support to a number of potential roles that may reflect why bulls with above average fertility have 3.5-fold higher concentrations of this protein.

2. Fertility-associated Antigen (FAA) and Tissue Inhibitor of Metalloproteinases-2 (TIMP-2)

Miller et al. (1990) isolated multiple proteins from bovine seminal fluid that possessed the ability to bind heparin and stimulate the zona pellucida-induced acrosome reaction of bovine sperm *in vitro*. Two of those proteins were identified to have a molecular mass of 31 kDa and 24 kDa. The 31-kDa protein was later "coined" fertility-

associated antigen (FAA; Bellin et al., 1998) with a shared homology to a DNase I-like protein (McCauley et al., 1999). The 24-kDa protein was later determined to be homologous to a bovine aortic tissue inhibitor of metalloproteinases-2 (TIMP-2; McCauley et al., 2001).

Both FAA and TIMP-2 are linked to bull fertility potential. Initial field studies that evaluated fertility of bulls used detergent extract of sperm and heparin-affinity chromatography to separate bulls based on protein profiles (Bellin et al., 1994). Bulls which contained the 31 kDa protein in sperm extracts were 17% more fertile than bulls which did not contain the 31-kDa protein (Bellin et al., 1994). A monoclonal antibody generated against purified heparin-binding proteins from seminal fluid, designated as M1 (Bellin et al., 1996), has been used to detect FAA and TIMP-2 in bovine semen (McCauley et al., 1999; 2001). By Western blot analysis, with the M1 antibody, Bellin and coworkers (1996; 1998) segregated bulls based on the presence or absence of the 31-kDa protein and conducted fertility trials. Bulls that possessed the antigen in sperm extracts were 14% higher in fertility than those lacking the antigen (Bellin et al., 1996; 1998). The previous fertility data describes the relationship of FAA (i.e., 31-kDa protein) on range bull fertility or under natural-mated conditions. When FAA positive and negative bulls were mated by artificial insemination (AI), FAA positive bulls were 16% more fertile than FAA-negative AI sires (Sprott et al., 2000).

The relationship of TIMP-2 to bull fertility has been established by a retrospective method (Dawson et al., 2002). Bulls who possessed TIMP-2 in detergent extracts of sperm were 13% more fertile than TIMP-2 negative bulls.

Although FAA and TIMP-2 positive bulls are more fertile than FAA and TIMP-2 negative herdmates the exact biological function of those proteins in relationship to bull fertility remains to be determined. Localization studies with the M1 antibody revealed antigen labeling over the acrosome and posterior head region of ejaculated bovine sperm (McCauley et al., 1996). That immuno-labeling pattern differed among bulls of varying fertility, but was not related to any cellular changes during capacitation and the acrosome reaction induced *in vitro* (McCauley et al., 1996). The latter finding may have been due to the inability to detect FAA and TIMP-2 as separate entities on the surface of sperm since the M1 antibody recognizes both fertility-related proteins.

As previously mentioned, FAA and TIMP-2 are high affinity heparin-binding proteins (Miller et al., 1990). Absence of those heparin-binding proteins on the surface of bull sperm appeared to be indicative of lower bull fertility. Similar findings do not exist in the literature for other livestock species. It is hypothesized that FAA and TIMP-2 bind to bovine sperm and thereby convey the capacitating effects of heparin *in vitro* or other heparin-like glycosaminoglycans *in vivo*. The exact mechanism(s) by which FAA/TIMP-2 bind to sperm and influence intra-cellular signaling pathways in bovine sperm remain to be determined.

IV. EXPLANATION OF DISSERTATION FORMAT

The chapters that follow represent studies that were performed to advance our knowledge of the fertility-related proteins, FAA and TIMP-2. A brief introduction along with the overall objectives of those studies is discussed below.

FAA and TIMP-2 can be used as predictors of bull fertility, however, little was known regarding their presence on sperm and whether they may be useful diagnostic markers for other species. Unique immuno-labeling patterns were identified for FAA and TIMP-2 using bull, ram and stallion sperm. It is hypothesized that FAA and TIMP-2 interact with heparin *in vitro* and other glycosaminoglycans present in the female reproductive tract to modulate capacitation. Thus, the presence of FAA and TIMP-2 on bovine sperm membranes was also monitored during the physiological induction of capacitation and acrosome reactions.

As mentioned above, FAA and TIMP-2 are predictors of bull fertility. Their presence or absence only in sperm membrane extracts established this relationship. Concentrations of FAA and TIMP-2 in seminal plasma have not been determined, nor if those concentrations were correlated to one another or reflective of bull fertility. Using enzyme-linked immunosorbent assays (ELISAs), the quantification of FAA and TIMP-2 in seminal plasma was performed. Previously, no immuno-detection method was available to determine absolute concentrations of FAA in a semen sample other than by densimetric analysis. The utility of the FAA ELISA will be exploited in numerous future studies by other individuals which will be a direct result of the assay developed from this dissertation objective.

The development of assays to assess fertility potential of bulls is limited to immuno-based detections methods using semen. The pitfall of this approach is that semen from bulls must be artificially collected which requires those bulls to be of reproductive age (~10-14 months old for most breeds). Due to the relationship of TIMP-2 to fertility, a further objective of this dissertation was to develop a detection method at the genomic level using amplified fragment length polymorphism (AFLP) methodology. Analysis of DNA for markers related to fertility would advance the degree of selection on fertility tremendously by removing current gender-biased testing. Such a test could reduce economic inputs into less fertile individuals. Preliminary data from the DNA fingerprint analysis had indicated there was a relationship to bull sterility. Exploitation of a genomic DNA marker for sterility in livestock would far exceed the economic value of any current immuno-based detection method for fertility.

The last study concludes by shifting to DNA-based methods to screen for an FAA template in stallion. Over the course of developing the studies herein, FAA was detected in semen from bulls, rams, boars, dogs and humans. However, FAA could not be detected (using two separate antibodies) in seminal fluid or sperm extracts from numerous fertile and sub-fertile stallions. Those observations were intriguing and warranted further investigation on a molecular level. Primers developed from the bovine FAA nucleotide consensus sequence were used to amplify a highly homologous gene product from the accessory sex glands of a fertile stallion. Those data describe an equine homologue to the bovine FAA gene and identified subtle differences in the translated nucleotide sequence which may partially explain the inability to detect FAA in stallion

semen using current monoclonal and polyclonal antibodies. Stallion spermatozoa may benefit substantially *in vitro* and *in vivo* from a therapeutic source of recombinant FAA if equine FAA is, in fact, less biologically active.

The following four chapters represent preprints of the studies discussed above in manuscript format for peer-reviewed journal submission once final corrections are made.

CHAPTER TWO

I. Localization of Two Fertility-Associated Seminal Heparin-binding Proteins to Sperm: A Comparison of Bull, Ram and Stallion[a]

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A. Abstract

Mammalian spermatozoa most undergo capacitation for successful fertilization to occur. The glycosaminoglycan, heparin, is a common constituent in chemically defined media which potentiates bovine sperm capacitation. Several heparin-binding proteins (HBPs) have been isolated from bovine semen that are hypothesized to mediate the interaction between heparin and bovine sperm. Of those HBPs, two have a high-affinity to bind heparin and have a relationship to bull fertility. One is a novel protein coined fertility-associated antigen (FAA) and the other is homologous to type-2 tissue inhibitor of metalloproteinases (TIMP-2). Multiple studies have implemented those proteins as predictors of bull fertility; however, no information is currently known about their specific location on bull, ram or stallion sperm or their fate during physiologically induced capacitation and the acrosome reaction. Therefore, our objectives were to 1.) Perform indirect immunofluorescence and localize both FAA and TIMP-2 to bull, ram and stallion sperm by using specific polyclonal antibodies directed against FAA and TIMP-2, and 2.) Monitor those fertility-related proteins through the process of bull sperm capacitation/acrosome reaction. Results demonstrated specific localization of FAA to the acrosome on bull and ram sperm. TIMP-2 was primarily localized to the posterior head region of ram and bull sperm while being specific to the stallion sperm acrosome. Following heparin-induced capacitation of bull sperm, FAA was absent following the acrosome reaction while TIMP-2 exhibited redistribution over the sperm head. These data indicated FAA and TIMP-2 bind to separate, unique regions on sperm and their localization patterns change as sperm become capacitated or acrosome reacted.

B. Introduction

Heparin, a glycosaminoglycan, facilitates sperm to undergo capacitation *in vitro* in several livestock species including bull (Parrish et al., 1988; 1989), ram (Slavik et al., 1990; Slavik and Fulka, 1991) and stallion (Farlin et al., 1993; Varner et al., 1993). Seminally derived heparin-binding proteins (HBPs) are hypothesized to mediate this interaction by binding to sperm at the time of ejaculation, potentiating the effects of heparin. In bulls, a series of HBPs ranging in size from 15-40 kDa possess a high-affinity to bind heparin (Miller et al. 1990), of these, a 31 kDa protein "coined" fertility-associated antigen (FAA; Bellin et al. 1998) was isolated, purified and characterized from bovine semen using a combination of RP-HPLC, SDS PAGE and Western blotting using a monoclonal antibody (M1Ab; McCauley et al., 1999).

Previous research had identified that approximately 50% of ejaculated bull sperm were labeled with the M1Ab with anterior (acrosomal) and posterior head labeling as the most frequent patterns (McCauley et al. 1996). Additionally, bull sperm retrieved by epididymal flushing were void of M1Ab labeling. However, the M1Ab was not mono-specific for FAA since it also cross-reacted with a 24 kDa and 27 kDa HBP in bovine semen (Bellin et al., 1996). Similar to FAA, the 24 kDa-protein was purified from seminal fluid, partially sequenced and found to be 90% identical to bovine aortic tissue inhibitor of metalloproteinases-2 (TIMP-2; McCauley et al., 2001). Tissue inhibitors of metalloproteinases (TIMPs) have been implicated in a number of reproductive processes including ovulation, fertilization, implantation and embryonic development. Both FAA

and TIMP-2 are produced and secreted by the bulbourethral, prostate and seminal vesicular glands (McCauley et al., 1999; 2001).

Immunoblot detection of FAA and TIMP-2 from bull sperm extracts has previously been used to segregate bulls and predict fertility. When naturally-mated, FAA positive bulls were approximately 19% more fertile than FAA-negative bulls (Bellin et al., 1994; 1996; 1998). Similarly, FAA-positive bulls were also 16% more fertile when their semen was used for artificial insemination (Sprott et al., 2000). In a retrospective analysis of bull fertility using the M1Ab that detects FAA and TIMP-2, TIMP-2 positive bulls were 13% more fertile than TIMP-2 negative bulls (Dawson et al., 2002). Collectively, the presence of FAA/TIMP-2 on sperm serves as a marker to identify fertility potential of bulls. However, no research has been conducted to document the presence of FAA or TIMP-2 on sperm from other livestock species.

Herein is the first report utilizing indirect immunofluorescence with anti-rFAA and anti-TIMP-2 polyclonal antibodies to separately localize FAA and TIMP-2 to bull, ram and stallion sperm. In addition, the fate of FAA and TIMP-2 during *in vitro* capacitation/acrosome reaction of bull sperm was evaluated to gain further understanding of the potential function of those proteins in regulating male fertility.

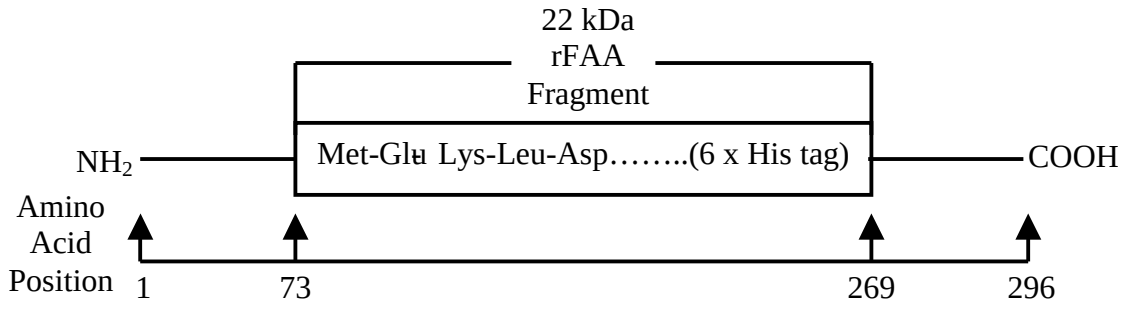
C. Materials and Methods

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless stated otherwise.

1. Production of Recombinant FAA Protein

RNA was extracted from the prostate of a sacrificed bull of known fertility using standard laboratory procedures. An RNeasy kit (Qiagen Inc., Chatsworth, CA) was used per manufacturer's directions to isolate RNA. Total RNA was quantified and stored at –80^{o} C. First-strand cDNA synthesis was performed using Superscript^{TM} II RNase H RT (GibcoBRL, Grand Island, NY). Bovine FAA was amplified from the cDNA products using primers that amplified a 600 bp product. The PCR products were cloned into the pCR2.10-TOPO vector (Invitrogen, Carlsbad, CA) and all positive clones were sequenced following electrophoresis. The partial cDNA (588 bp) encoding for the recombinant fragment was re-cloned into the pCR T7/CT-TOPO inducible expression vector (Invitrogen). Transformation and expression were performed using One-shot BL21(DE3) cells (Invitrogen). Three hours post-induction, cells were harvested and protein extracted. Crude protein extracts were applied to a heparin-affinity column (Bio-Rad, Hercules, CA) connected to an in-line peristaltic pump at a flow rate of 1 ml/min using a TCA buffer (40 mM Tris, 2 mM $CaCl_2$, 200 *u*M PMSF, 0.01% NaN_3; pH=7.4). Absorbance was measured at 280 nm with a UA-5 absorbance detector (ISCO, Lincoln, NE). The column was eluted with 2 M NaCl in TCA and protein desalted and concentrated. Protein concentration was determined using the Bio-Rad D_c protein assay kit (Bio-Rad) using bovine serum albumin (BSA) as the standard. All separations took place at 4^{o} C, and protein was then frozen and lyophilized. Lyophilized powder was further purified by reversed-phase HPLC similar to the native conditions defined by

McCauley et al. (1999). Mass spectral analysis resulted in a 22-kDa protein. The illustration below compares the native to the recombinant FAA fragment.



2. Polyclonal Recombinant FAA Antiserum Production

Rabbit antiserum production was performed by Strategic Biosolutions (Windham, ME) on a contractual basis following standard immunization protocols using the recombinant FAA fragment. Rabbit sera collected pre- and post-immunization were analyzed for cross reactivity using immunoblots of bovine sperm protein extracts. The original monoclonal antibody M1 (M1Ab) was used as the reference standard (Bellin et al., 1996). Rabbits successfully developed antibodies against the rFAA fragment, and those antisera recognized the native 31 kDa, fertility-associated antigen in bovine sperm extracts (McCauley et al., 2004).

3. Polyclonal TIMP-2 Antiserum

Rabbit polyclonal TIMP-2 antiserum was graciously donated by Dr. William G. Stetler-Stevenson from the National Cancer Institute (Bethesda, MD) previously described in Stetler-Stevenson et al. (1990).

4. Indirect Immunofluorescence of FAA and TIMP-2 on Sperm

Frozen sperm from bulls, rams and stallions were washed in 50 mM phosphate buffered saline (PBS; pH=7.2) and centrifuged for 3 min at 400 x g in 1.5 ml microcentrifuge tubes. After three washes, sperm were resuspended in 95% ethanol and incubated for 30 min on ice. Ethanol treated sperm were centrifuged and resuspended in PBS and stored at 4^{o} C. Sperm were mounted on slides and air-dried in duplicate. Nonspecific binding sites were blocked with 10% normal goat serum for 45 min at room temperature. Slides were rinsed in PBS and dilutions of primary antibodies (rFAA, 1:500; TIMP-2, 1:100) were added and incubated for two h at room temperature. After multiple washes in PBS, slides were incubated with FITC-conjugated goat anti-rabbit secondary antibodies (1:160; Jackson ImmunoResearch, Inc., West Grove, PA) containing 5 ug/ml of 4',6-diamidino-2-phenylindole (DAPI) for 45 min. Pre-immune or normal rabbit sera and secondary antibodies alone served as controls. Slides were washed and 25 ul anti-fade mounting medium (VectaShield, Vector Laboratories, Inc., Burlingame, CA) was applied. Slides were analyzed with a Leitz Diaplan microscope equipped for epifluorescence and images were captured with linked Alpha Imager™ software (Alpha Innotech Corporation, San Leandro, CA). Images were then transferred into Adobe PhotoShop 7.0 for publication purposes.

5. Bovine Sperm Capacitation Assay

Cryopreserved bull semen was used to determine if FAA or TIMP-2 localization patterns were altered following capacitation/acrosome reaction. Semen samples from three separate collections from three bulls of known fertility were used in replicate.

Sperm were thawed at 38^{o} C for 15 sec and washed three times using 1-ml of TALP medium (McCauley et al., 1996). After the final wash, sperm were equally divided and assigned to the following treatments: 1.) Untreated (T=0 h); 2.) 3-h TALP only; 3.) 3-h TALP+10 *u*g/ml heparin (sodium salt from porcine intestinal mucosa; Scientific Protein Laboratories, Waunakee, WI) for the evaluation of sperm capacitation; and 4.) 3-h TALP+10 *u*g/ml heparin followed by addition of 100 ug/ml lysophosphatidylcholine (LPC; Type III from bovine liver) to induce the acrosome reaction for 15 min. The percent of sperm undergoing the acrosome reaction (%AR) was scored at T=0 and following each treatment by counting 100 sperm/slide using FITC conjugated *Pisum sativum agglutinin* (FITC-PSA; 50 ug/ml) lectin as described by Cross et al. (1986). Only those sperm that exhibited even fluorescence over the acrosomal region were considered intact. In addition, indirect immunofluorescence was performed using FAA and TIMP-2 polyclonal antisera at all time points across treatments as described previously. One hundred sperm per slide (three localization patterns) were evaluated for slides labeled with rFAA antiserum and 200 sperm per slide (four localization patterns) for sperm labeled with TIMP-2.

D. Statistical Analysis

A model was fit to the capacitation data for three independent data sets which independently examined %AR, FAA localization patterns and TIMP-2 localization patterns. Data were analyzed using PROC GENMOD with the Wald criteria for categorical data in Statistical Analysis System (SAS, 1999). The response variable, sperm count, was assumed to be Poisson distributed and a model was fit using the PROC

GENMOD procedure of SAS with a logarithmic link function. The models included fixed effects of treatment (n=4) and sperm fluorescence pattern for %AR (acrosome reacted or acrosome intact), FAA (pattern A, B, or C) and TIMP-2 (pattern A, B, C or D). Ejaculates within bull were considered repeated subjects in each model. Least squares means (lsmeans) were computed for all treatment response variables. An alpha level of $P \leq 0.05$ was used to control for Type I errors.

Models for the analysis of %AR, FAA and TIMP-2 patterns were like below:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

where,

Y_{ijk} = sperm count

μ = population mean

α_i = i^{th} treatment effect (control, TALP, heparin, heparin+LPC)

β_j = j^{th} pattern effect (acrosome intact or reacted; localization pattern A, B, C and/or D)

$(\alpha\beta)_{ij}$ = interaction between i^{th} treatment and j^{th} pattern effect

ε_{ijk} = error term

E. Results

1. Immunolocalization of FAA to Bull, Ram and Stallion Sperm

Using indirect immunofluorescence, FAA was localized specifically to the acrosome of bull (Figure 2.1) and ram (Figure 2.2) sperm. No cross-reactivity was observed on stallion sperm (Figure 2.3). Pre-immune rabbit serum (1:500) served as the

control for all species. Secondary antibodies alone served as an additional control with no cross-reactivity observed (data not shown).

2. Immunolocalization of TIMP-2 to Bull, Ram and Stallion Sperm

TIMP-2 was localized to bull, ram and stallion sperm. The majority of TIMP-2 labeling was observed over the posterior head region of bull (Figure 2.4) and ram (Figure 2.5) sperm. Some bull sperm exhibited uneven fluorescence over the anterior acrosome. In contrast, stallion sperm (Figure 2.6) possessed intense TIMP-2 cross-reactivity over the entire acrosomal cap region. Normal rabbit serum (1:100) served as the control for all species. Secondary antibodies alone served as an additional control with no cross-reactivity observed (data not shown).

3. Capacitation/Acrosome Reaction and Localization of FAA & TIMP-2 to Bull Sperm

Cryopreserved bull sperm were cultured in TALP (control), TALP+heparin, or TALP+heparin+LPC as described previously to evaluate FAA and TIMP-2 localization patterns prior to and following the induction of capacitation/acrosome reaction. The number of sperm undergoing the acrosome reaction was evaluated using FITC-PSA lectin (Figure 2.7). Sperm were categorized as acrosome reacted (absence of fluorescence over the acrosome) or acrosome intact (presence of even fluorescence over the acrosome) and analyzed using the statistical model previously described. After three hours of culture in the presence of heparin and the addition of LPC, higher numbers of sperm were acrosome reacted ($P<0.0001$). No difference was detected for the incidence of acrosome-reacted sperm cultured in TALP or TALP+heparin ($P>0.6$).

FAA localization patterns were evaluated following the capacitation/acrosome reaction assay. Figure 2.8a includes the lsmean±SE of sperm labeled with FAA at T=0, and after 3-h culture for each respective treatment. Three primary FAA localization patterns were observed and those patterns are identified in Figure 2.8b as pattern A, B or C, respectively. A significant ($P<0.0001$) treatment by pattern interaction was observed. At 0-hour, approximately 65% of bull sperm labeled with intense fluorescence over the acrosomal cap region (pattern A). Following the three-hour culture, there was a reduction ($P<0.001$) in the number of sperm exhibiting pattern A labeling in all treatments. No difference ($P=0.37$) was detected in pattern A for TALP or TALP+heparin treatments at the end of 3-h. Furthermore, no difference ($P=0.13$) was observed at any time point for pattern B. In comparing Figures 2.7 and 2.8, after sperm had undergone the acrosome reaction (Heparin+LPC; see Figure 2.7), there was a significant ($P<0.001$) increase in sperm categorized as pattern C (no label; Figure 2.8). The reduction in number of sperm exhibiting acrosomal cap labeling (pattern A) appeared after sperm had undergone the acrosome reaction (Heparin+LPC). This suggested that FAA localization patterns on sperm were altered by the acrosome reaction but not capacitation.

Additionally, TIMP-2 fluorescent patterns were also evaluated following capacitation/acrosome reaction. Figure 2.9a illustrates the lsmean±SE of sperm labeled with TIMP-2 at T=0, and after 3-h culture for each respective treatment. Four primary TIMP-2 localization patterns were observed and those patterns are identified in Figure 2.9b as pattern A, B, C or D, respectively. A significant ($P<0.0001$) treatment by pattern

interaction was observed. The primary pattern A (posterior head only) did not differ ($P>0.8$) at 0-hour or after sperm were cultured 3-h in TALP (non-capacitated sperm). However, a reduction in sperm with posterior head TIMP-2 labeling was apparent when sperm were cultured in heparin ($P<0.05$), but no difference ($P>0.7$) was observed after addition of the fusogenic reagent LPC to induce the acrosome reaction. Pattern C labeled sperm (posterior head and punctate acrosome) was highest ($P<0.05$) after culture in heparin, although no difference ($P>0.2$) was detected between heparin and heparin+LPC treated sperm. Redistribution of TIMP-2 occurred on sperm during culture while total absence of TIMP-2 labeled sperm (pattern D) was the least prevalent pattern observed across all treatments. The redistribution of TIMP-2 on bull sperm appeared to be associated with capacitation (heparin treatment) and not a consequence of the induced acrosome reaction (heparin+LPC).

F. Discussion

The presence of heparin-binding proteins (HBPs) in mammalian semen is well established. Previous work has identified two specific sperm-associated HBPs, FAA and TIMP-2, and their presence on sperm in relation to bull fertility has been described. In addition, those proteins are absent on epididymal sperm and cross-reactivity with the M1Ab appears after sperm undergo normal ejaculation. Using the M1Ab, the cross-reactivity is observed on the acrosomal cap and posterior head regions (McCauley et al., 1996). Since those proteins bind to sperm and are related to bull fertility, this study evaluated their presence on sperm from bulls, rams and stallions using mono-specific polyclonal antibodies for FAA and TIMP-2.

For bulls and rams, FAA was specifically localized to the acrosome of sperm using indirect immunofluorescence. The mono-specificity of the anti-rFAA antibody for bovine FAA has previously been demonstrated by McCauley et al. (2004). In addition, FAA was not detected on stallion sperm by immunofluorescence. This finding is additionally supported by the lack of immunoblot detection of FAA using sperm extracts and seminal plasma from multiple fertile stallions (data not shown). Bull and ram sperm respond similarly when capacitated with a given dose of heparin (i.e. 10 ug/ml). A bull and stallion heparin-induced capacitation comparison study by Farlin et al. (1993) found stallion sperm required in excess of ≥50 ug/ml of heparin to become capacitated above control values. The efficacy by which heparin influences stallion sperm capacitation in concert with the absence of the high-affinity HBP, FAA, is intriguing. FAA may, in fact, be absent in stallions, or an altered form is produced and secreted which could have a lower affinity for heparin. Further work is needed to elucidate the reasons why stallion sperm respond more variably to heparin *in vitro* compared to other livestock species.

Immunolocalization of TIMP-2 was also performed for all species. TIMP-2 was primarily localized to the posterior head of bull (Figure 2.4) and ram (Figure 2.5) sperm, but exclusively found on the acrosomal cap of stallion sperm (Figure 2.6). The presence of TIMP-2 in seminal plasma has been documented for bulls (Liberda et al., 2001; McCauley et al., 2001; Calvete et al., 1996), humans (Shimokawa et al., 2003; Baumgart et al., 2002a and 2002b) and in testicular and epididymal fluids from rams and stallions (Metayer et al., 2002). To our knowledge, this is the first report providing direct evidence that TIMP-2 was present on bull, ram and stallion sperm surfaces.

Since FAA and TIMP-2 are related to bull fertility and both antigens are present in distinct regions on bovine sperm, the fate of FAA and TIMP-2 after heparin-induced capacitation was ascertained. FAA binding patterns for bull sperm were not affected by culture in media alone or under capacitating conditions; however, the induction of the acrosome reaction by LPC resulted in a significant increase of sperm no longer exhibiting the epitope for FAA. During capacitation, the plasma membrane begins to reorganize and prepare for fusion with the underlying outer acrosomal membrane. Following the acrosome reaction, the majority of the plasma membrane and outer acrosomal membrane are lost. Therefore, these data suggest that the epitope for FAA appears to be present on either the plasma or outer acrosomal membrane (or both). Although a possible, progressive redistribution of FAA from acrosomal cap (Pattern A), to underlying plasma membrane (Pattern B), to absence of labeling (Pattern C) may be the means by which FAA disappears, we are unable to conclude this until immuno-electron microscopy is performed. Moreover, it is intriguing that approximately 50% of sperm no longer label with anti-FAA when those populations of sperm displayed over 50% acrosome reacted cells. A dual-labeling technique to identify acrosomal status (intact or acrosome reacted) and FAA presence or absence needs to be performed to validate whether sperm identified as “pattern C” are truly acrosome reacted.

In contrast to FAA, TIMP-2 appears to undergo redistribution on the sperm cell without complete epitope loss following the acrosome reaction. Over one-half of bull sperm exhibited the epitope only on the posterior portion of the sperm head at 0-h and following 3-h culture in TALP (control). When heparin was present, there was a

reduction in posterior head only labeling pattern and a significant increase in sperm exhibiting a punctate acrosome/posterior head (Pattern C). This apparent redistribution of TIMP-2 during capacitation may be due in part to their physiological interactions with the matrix metalloproteinases (MMPs), a family of proteolytic enzymes. TIMPs are known to regulate membrane-bound MMPs by inhibiting the cleavage or conversion of pro-MMP form to the active MMP form. MMPs have been localized to the acrosome and midpiece of normal and abnormal human sperm (Buchman-Shaked et al., 2002). Significance of this finding is currently unknown, but would suggest if proteolytic activity does occur by MMPs on the acrosome of sperm, that TIMPs may play a role in modulating MMP activity. It may be the migration of TIMP 2 from posterior head to acrosomal region that regulates the rate of membrane proteolysis or fusion, protecting sperm from premature capacitation or acrosome reaction. Co-localization of TIMP-2 and candidate MMPs should further elucidate the interaction between tissue inhibitors and matrix metalloproteinases on sperm.

In conclusion, this study demonstrates unique binding sites for FAA and TIMP-2 on bull, ram and stallion sperm. The binding of specific HBPs to sperm may be required for the capacitating effects of heparin to be conveyed. Future studies should evaluate sperm from high and low fertility males, as objective sperm-specific markers are needed to assess male fertility in other livestock species.

Acknowledgments

The authors would like to extend their sincere appreciation and recognition to Dr. H.M. Zhang and M.E. Bellin for the production and purification of the recombinant FAA

protein as described in the Materials and Methods. In addition, appreciation is extended to ReproTec, Inc. (Tucson, AZ) for the donation of the polyclonal anti-rFAA sera that was developed through funding by the USDA-SBIR grant program and to Dr. W.G. Stetler-Stevenson from the National Cancer Institute (Bethesda, MD) for the polyclonal anti-TIMP-2 sera for this research.

Figure 2.1. Immunolocalization of Fertility-Associated Antigen (FAA) to bovine sperm using FITC-conjugated secondary antibodies (column A) with corresponding DAPI (middle panel) and bright field (bottom panel) image. Column B represents control pre-immune rabbit serum with corresponding DAPI and bright field image.

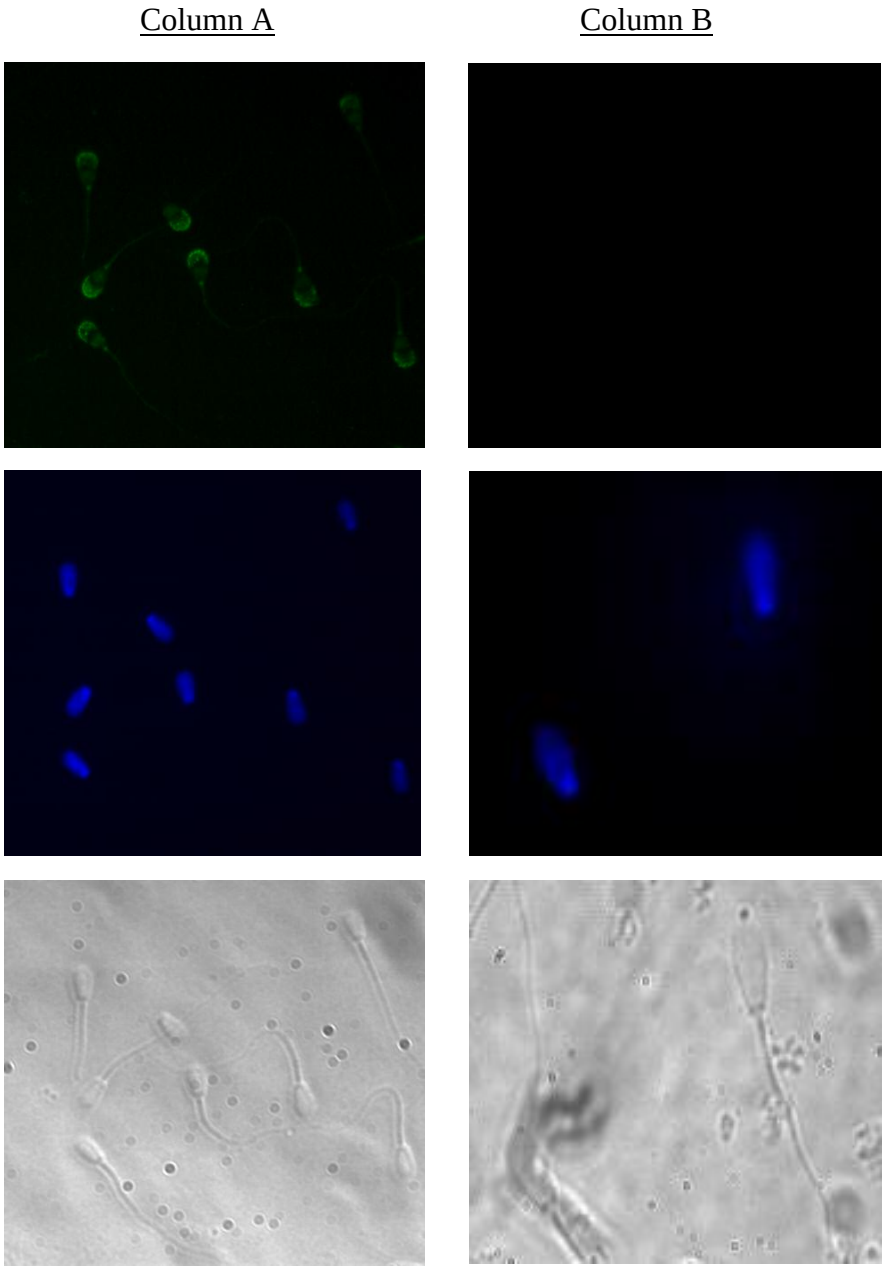


Figure 2.2. Immunolocalization of Fertility-Associated Antigen (FAA) to ram sperm using FITC-conjugated secondary antibodies (column A) with corresponding DAPI (middle panel) and bright field (bottom panel) image. Column B represents control pre-immune rabbit serum with corresponding DAPI and bright field image.

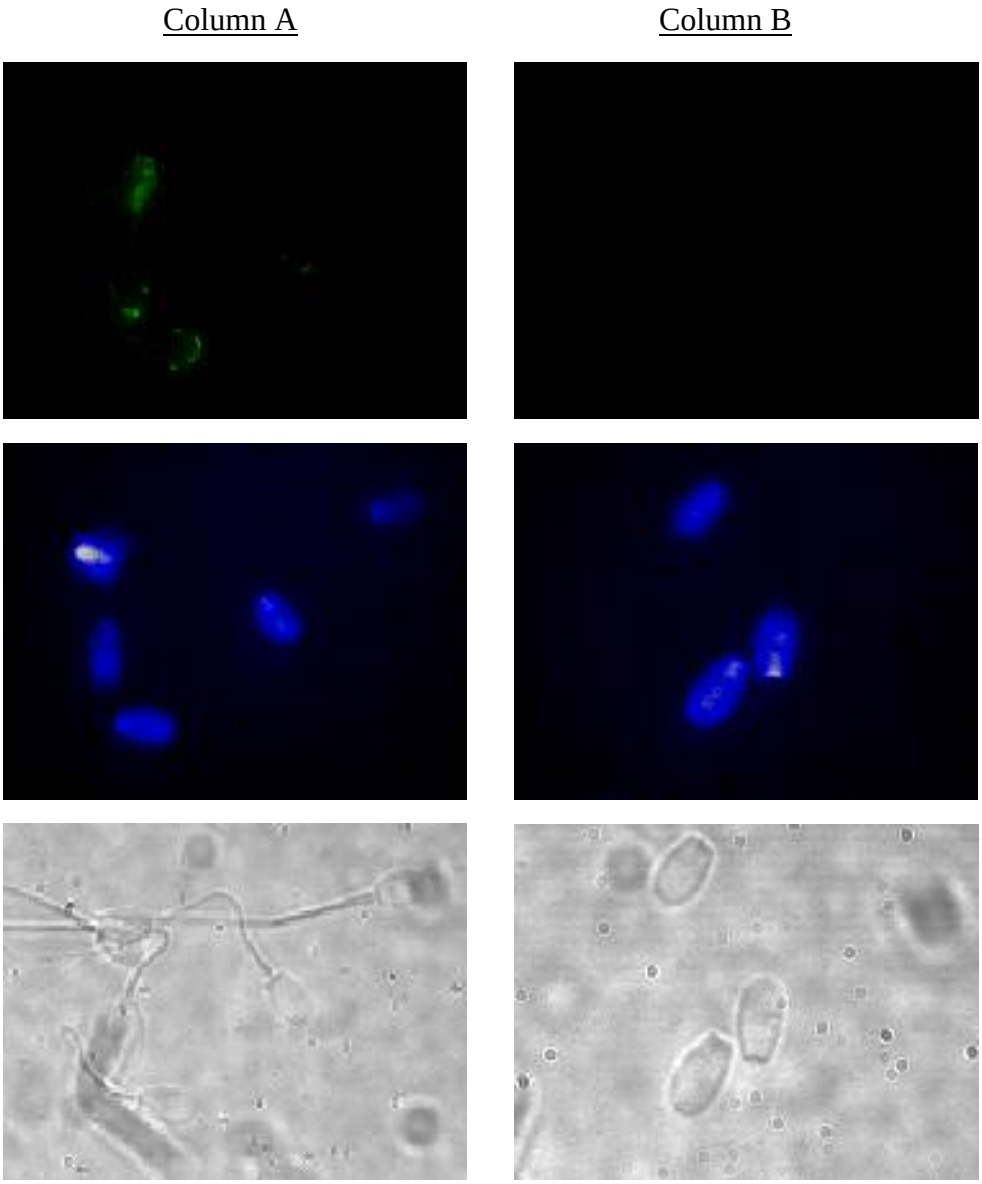


Figure 2.3. Lack of immunolocalization of Fertility-Associated Antigen (FAA) to equine sperm using FITC-conjugated secondary antibodies (column A) with corresponding DAPI (middle panel) and bright field (bottom panel) image. Column B represents control pre-immune rabbit serum with corresponding DAPI and bright field image.

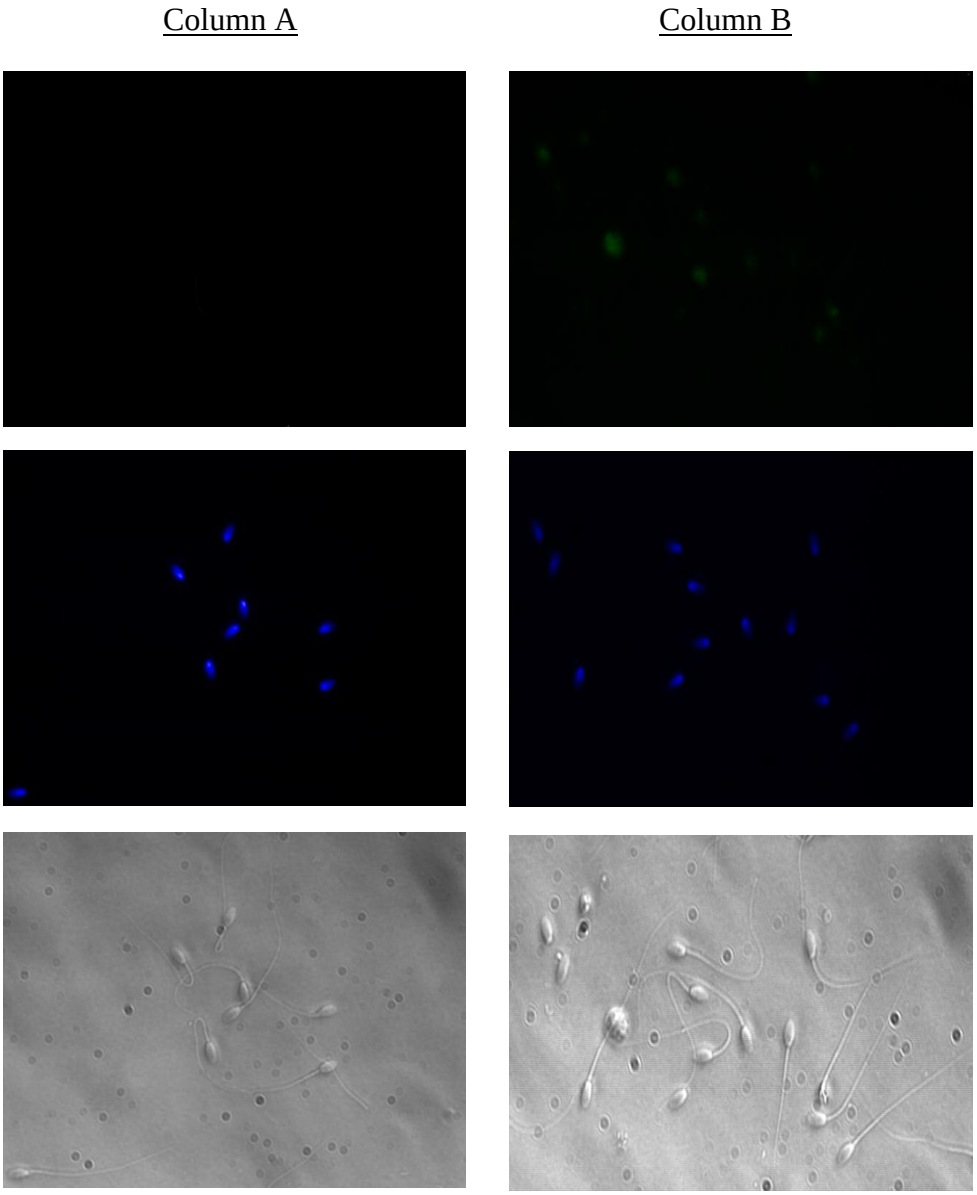


Figure 2.4. Immunolocalization of Type-2 Tissue Inhibitor of Metalloproteinases (TIMP-2) to bovine sperm using FITC-conjugated secondary antibodies (column A) with corresponding DAPI (middle panel) and bright field (bottom panel) image. Column B represents control pre-immune rabbit serum with corresponding DAPI and bright field image.

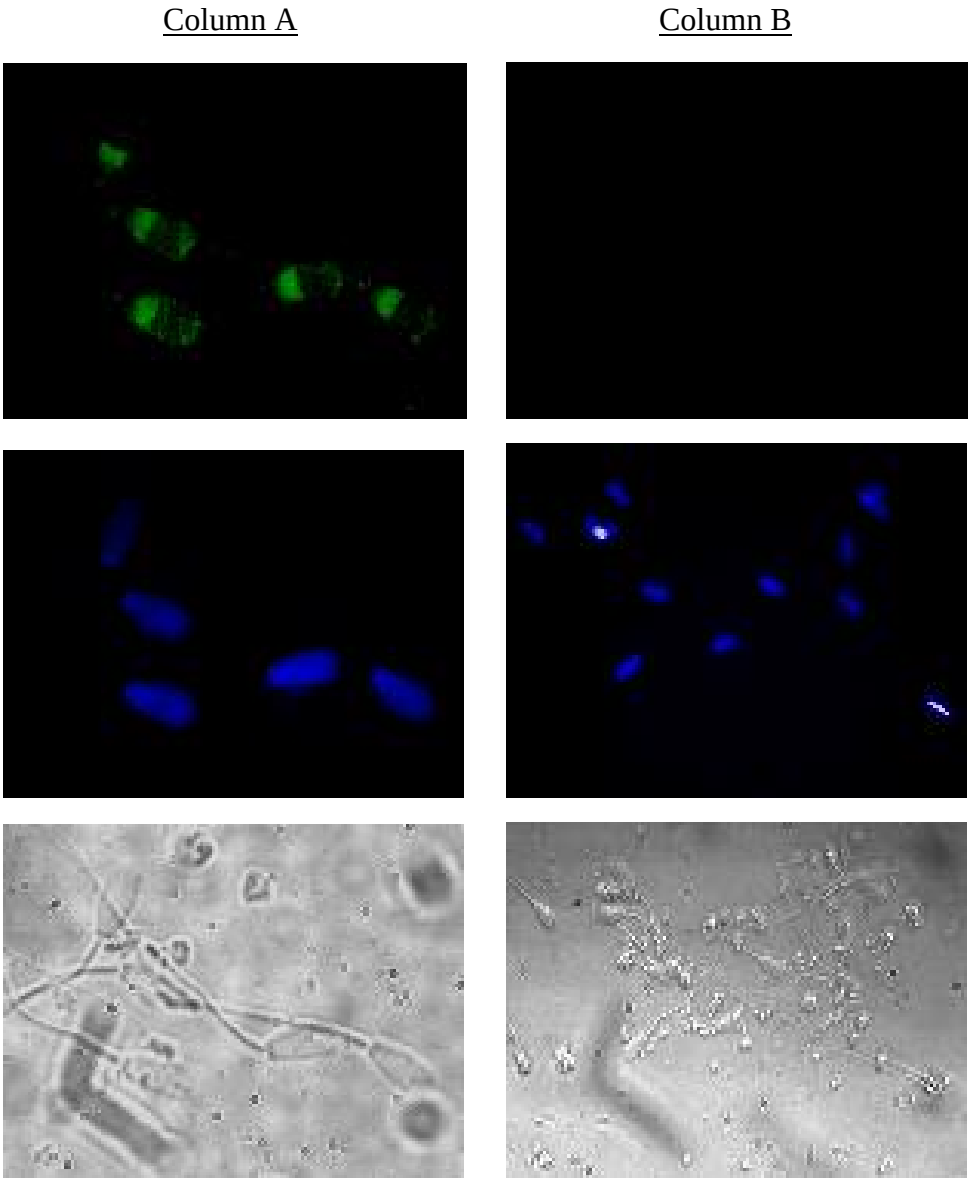


Figure 2.5. Immunolocalization of Type-2 Tissue Inhibitor of Metalloproteinases (TIMP-2) to ram sperm using FITC-conjugated secondary antibodies (column A) with corresponding DAPI (middle panel) and bright field (bottom panel) image. Column B represents control pre-immune rabbit serum with corresponding DAPI and bright field image.

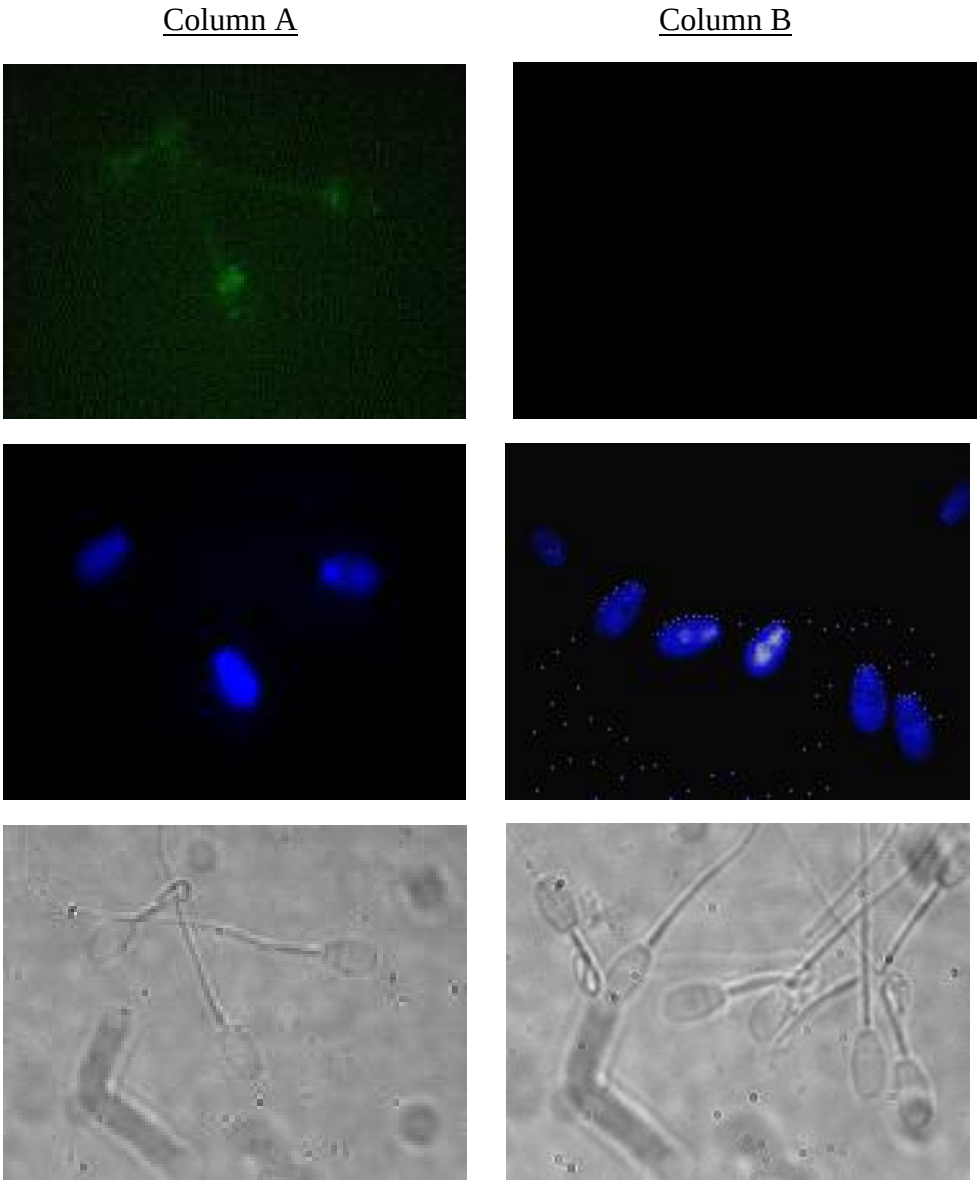


Figure 2.6. Immunolocalization of Type-2 Tissue Inhibitor of Metalloproteinases (TIMP-2) to equine sperm using FITC-conjugated secondary antibodies (column A) with corresponding DAPI (middle panel) and bright field (bottom panel) image. Column B represents control pre-immune rabbit serum with corresponding DAPI and bright field image.

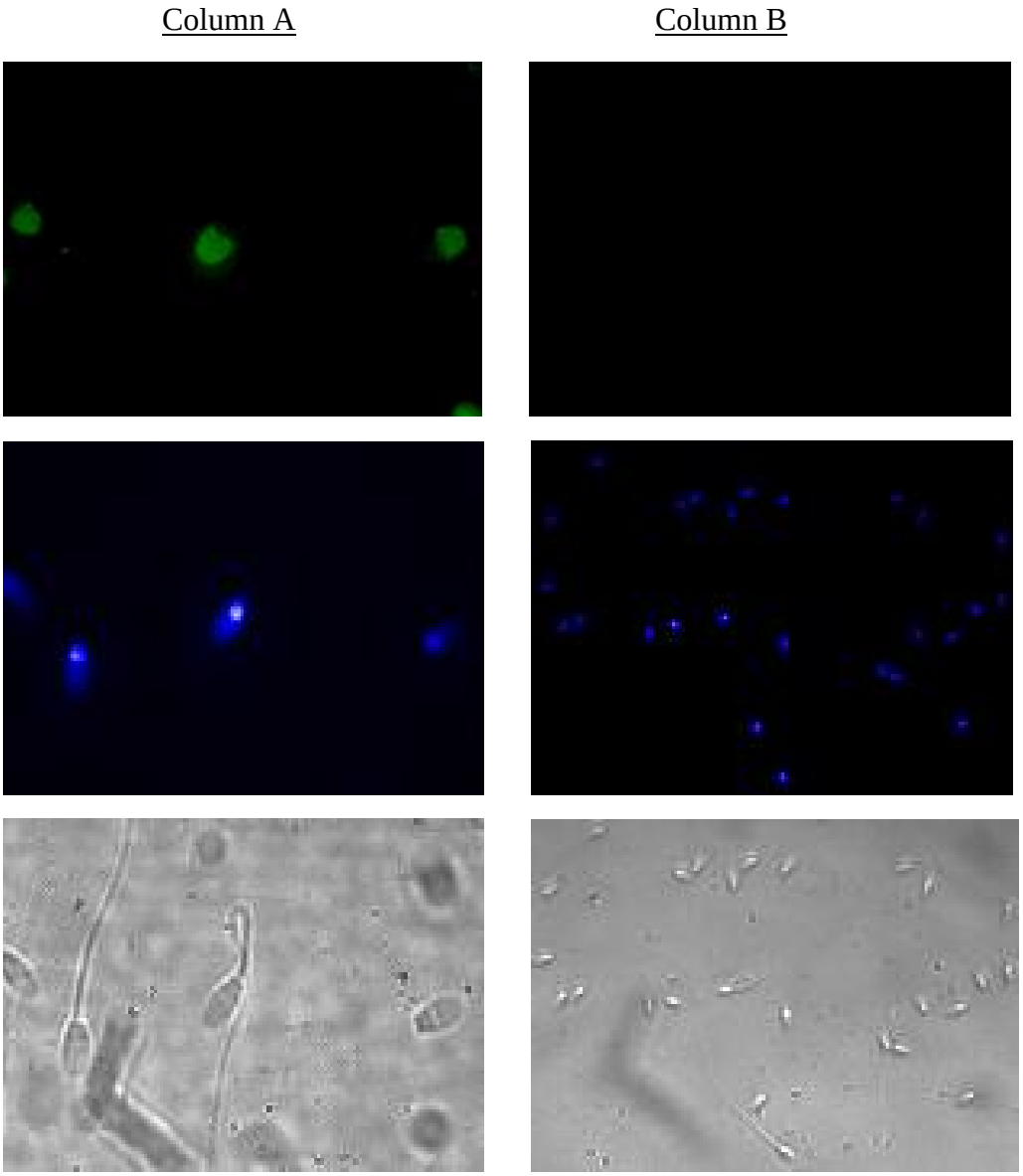
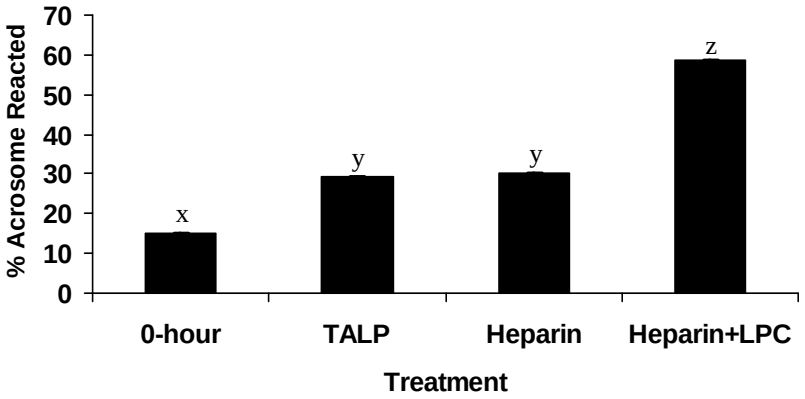


Figure 2.7. Percent (%) of acrosome-reacted (AR) spermatozoa determined at O h and following a 3 h incubation in TALP media, TALP+heparin and TALP+heparin with exposure to lysophosphatidylcholine (LPC) for 15 min after the 3 h incubation. AR were scored using FITC conjugated PSA lectin.

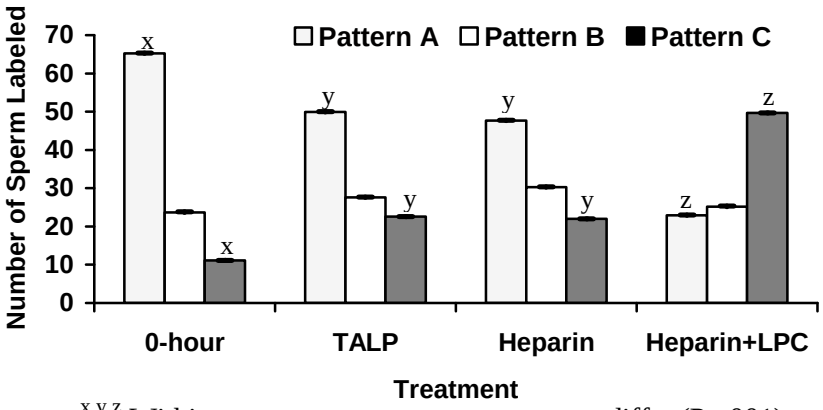


[x,y,z] Values with different superscripts differ (P<.0001)

* Values represent lsmeans±SE from 100 sperm per slide counted using three separate ejaculates from each of three bulls (N=9).

Figure 2.8. Fertility-associated antigen (FAA) immunolocalization patterns are altered as bovine spermatozoa undergo the acrosome reaction in vitro.* Mean±SE (a.) number of sperm labeled as pattern A, B and C for each respective treatment. b.) Sperm were grouped according to labeled pattern A (acrosomal cap), B (outline acrosomal cap) or C (no label).

a.)



x,y,z Within pattern across treatments, means differ (P<.001). Significant (P<.0001) treatment by pattern interaction.

* Data represent lsmeans±SE from 100 sperm per slide counted using three separate ejaculates from each of three bulls (N=9).

b.)

Pattern A Pattern B Pattern C

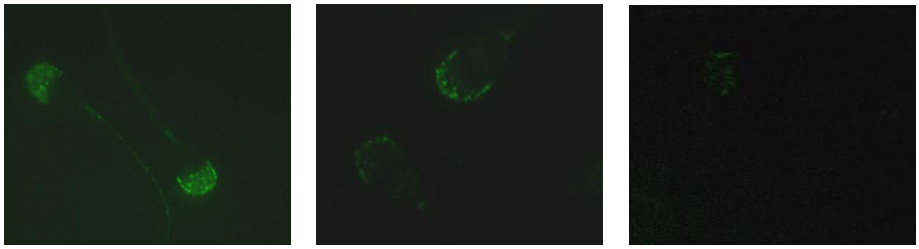
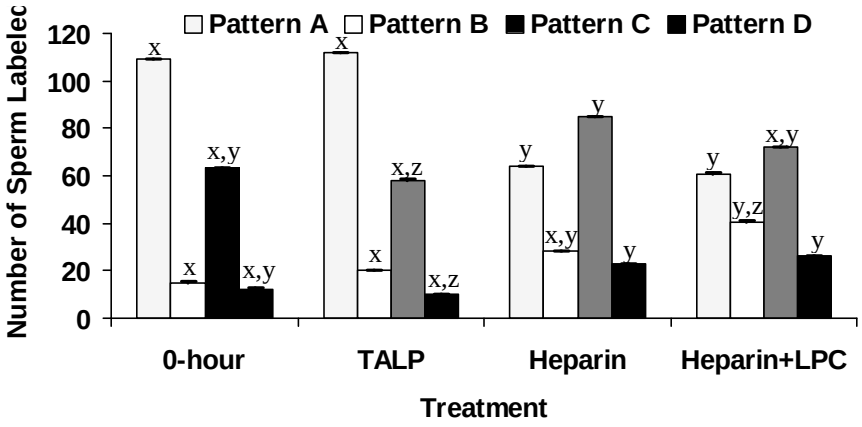


Figure 2.9. Type-2 Tissue Inhibitor of Metalloproteinases (TIMP-2) immunolocalization patterns are altered as bovine spermatozoa undergo capacitation in vitro.* Mean±SE (a.) number of sperm labeled as pattern A, B, C and D for each respective treatment. b.) Sperm were grouped according to labeled pattern A (posterior head), B (posterior head and acrosome), C (posterior head and punctate acrosome) and D (no label).

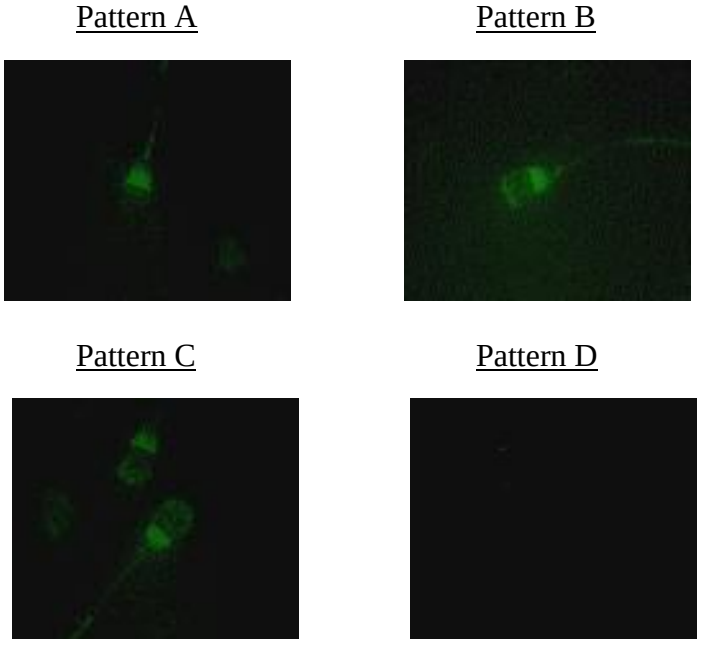
a.)



[x,y,z] Within pattern across treatments, means differ (P<.01). Significant (P<.0001) treatment by pattern interaction.

* Data represent lsmeans±SE from 200 sperm per slide counted using three separate ejaculates from each of three bulls (N=9).

b.)



CHAPTER THREE

I. Quantification of Two Bovine Fertility-associated Proteins in Seminal Fluid by Enzyme-Linked Immunosorbent Assay (ELISA)[a]

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A. Abstract

Fertility-associated antigen (FAA) and tissue inhibitor of metalloproteinases-type 2 (TIMP-2) are two peptides that bind heparin. Their presence has been detected on ejaculated bovine sperm and in seminal fluid using monoclonal and polyclonal antibodies, but not on epididymal sperm. Unlike ejaculated bovine sperm, epididymal sperm do not respond to heparin when cultured *in vitro*. However, purified fractions of FAA and TIMP-2 have been added to epididymal sperm and those sperm then become responsive to heparin. In addition, the presence or absence of FAA and TIMP-2 on sperm membranes can be used to predict bull fertility. Traditionally, immuno-detection of FAA and TIMP-2 is performed by Western blot analysis of protein extracts isolated from sperm. This method is cumbersome and quantification of FAA and TIMP-2 on immunoblots by densimetric analysis can be difficult.

Therefore, an enzyme-linked immunosorbent assay (ELISA) was developed for the detection and quantification of FAA in seminal plasma. Also utilized was a commercially available ELISA for TIMP-2. A fertility trial was conducted to determine if concentrations of FAA or TIMP-2 in seminal plasma, alone, could be used as a predictor of bull fertility. FAA concentrations in seminal plasma ranged from 0 to 37.9 ug/ml, while TIMP-2 concentrations ranged from 0.92 to 2.41 mg/ml. With increasing age, bulls were more prolific in terms of calves sired based upon DNA-based verification of calf parentage.

B. Introduction

Bulls with identical physical semen characteristics still vary greatly in actual fertility when mated naturally or when they are used in artificial insemination programs. Therefore, objective semen markers are needed to identify those bulls which are more fertile (Foote, 2003). To date, two heparin-binding proteins (HBPs) have been identified in bovine semen that can be used as predictors of bull fertility. Those proteins are fertility-associated antigen (FAA; Bellin et al., 1998; McCauley et al., 1999), and tissue inhibitor of metalloproteinases-2 (TIMP-2; McCauley et al., 2001). Heparin is a common physiological reagent used in chemically defined media to capacitate bovine sperm *in vitro* (Parrish et al., 1988 and 1989), and heparin-like materials can be used as probes to monitor fertility differences in bulls (Ax and Lenz, 1987). Sperm from those higher fertility bulls are more susceptible to capacitation *in vitro* (Ax et al., 1985), and display a higher binding affinity for heparin in ligand-binding assays (Marks and Ax, 1985).

FAA and TIMP-2 are hypothesized to interact with heparin and thereby convey the capacitation-like effects necessary for successful fertilization to occur. Both FAA and TIMP-2 are secreted from the prostate, bulbourethral and seminal vesicular glands of bulls (McCauley et al., 1999 and 2001). FAA appears to be a unique protein with shared homology to the DNase-I family (McCauley et al., 1999). DNase-I activity has been reported in semen, and it is present in human prostatic secretions (Yasuda et al., 1993) and developing spermatogonia and spermatocytes (Stephan et al., 1996). However, the role of DNase-I family members in male reproduction is not well understood. In contrast, TIMP-2 is a well-characterized inhibitor of the matrix metalloproteinases (MMPs), a

large family of proteolytic enzymes (reviewed by Brew et al., 2000; Sternlicht and Webb, 2001). The presence of TIMP-2 has been detected in male reproductive tracts from numerous domestic animals (Metayer et al., 2002) and in seminal fluid from normal and sub-fertile humans (Baumgart et al., 2002a and 2002b). TIMP-2 has been established as a major bovine seminal plasma protein (Calvete et al., 1996). Immunodetection of both FAA and TIMP-2 has been performed with a monoclonal antibody M1 (M1Ab; Bellin et al., 1996). A localization study identified that the M1Ab bound to ejaculated bovine sperm but not to epididymal sperm (McCauley et al., 1996).

Traditionally, bulls are characterized based on the presence or absence of FAA and TIMP-2 on their sperm membranes by Western blot detection. In a series of field trials conducted by Bellin et al. (1994; 1996 and 1998), which used only bulls that passed a breeding soundness exam (BSE), bulls which produced sperm with detectable amounts of FAA were 19% more fertile than bulls that did not possess FAA in sperm membrane extracts. A retrospective analysis of range bull fertility was also performed with TIMP-2, and that study identified bulls that were TIMP-2 positive were 13% more fertile than bulls who produced sperm without detectable TIMP-2 (Dawson et al., 2002).

None of the above studies have established the concentration of FAA or TIMP-2 in bovine semen, or evaluated whether amounts of FAA/TIMP-2 in seminal fluid alone corresponded to bull fertility. Therefore, our objectives were to: 1) Develop an FAA enzyme-linked immunosorbent assay (ELISA) using recombinant FAA polyclonal antibodies that have previously been shown to be mono-specific for bovine FAA (McCauley et al., 2004) to accurately assess the concentration of FAA in seminal plasma;

2) Utilize a commercially available TIMP-2 ELISA to establish TIMP 2 concentrations in bovine seminal plasma; and 3) Determine if the concentration of FAA and TIMP-2 in seminal fluid alone was related to bull fertility.

C. Materials and Methods

1. Fertility Trial

A fertility trial was conducted over three consecutive breeding seasons using only bulls that were qualified as potential satisfactory sires following a breeding soundness exam (BSE). At the onset of the trial when semen was collected for the BSE and ELISAs, bulls ranged in age from less than one (<1) to three-years of age. Semen collected during the BSE was frozen and stored at -20^{o} C. All bulls were maintained at a constant ratio of one bull per 25 cows for a 60-d breeding season in randomly assigned multi-sire pastures. Blood samples were taken on all potential sires and all of the resulting male offspring (one-half of the calf crop). For each potential sire, it was assumed there was a 50% chance to sire a male calf and 50% chance to sire a female calf. This limited the number of calves to undergo parental determination testing and reduced the cost of that testing.

All parental testing was performed on a contractual basis by Maxaam Analytics, Inc. (Guelph, Canada). A total of 15 microsatellite markers was used for parentage determination.

2. Peroxidase Conjugation of Recombinant Fertility-associated Antigen (rFAA) Antiserum

Affinity purified anti-rFAA antibody from Midland, Bioproducts (Boone, IA) was peroxidase conjugated using EZ-Link™ Plus Activated Peroxidase kit (Pierce, Rockford, IL) per manufacturer's directions. Dextran desalting columns (Pierce) were used to purify peroxidase labeled anti-rFAA. One-ml fractions were collected and concentration (OD_{280}) determined. Fractions containing peroxidase conjugated anti-rFAA were further concentrated using Centricon 30 spin columns (Millipore Corporation, Bedford, MA). Glycerol was added at a final concentration of 50%. Aliquots of peroxidase conjugated anti-rFAA were stored at -20^{o} C.

3. Enzyme-linked Immunosorbent Assay (ELISA) for Fertility-associated Antigen (FAA)

The concentration of FAA in bovine seminal plasma was determined by capture ELISA. Semen collected by electroejaculation from 46 bulls was used in the experiment. Five hundred microliters of neat semen from each individual bull were centrifuged at 10,000x g for 10 min at 4^{o} C. Following centrifugation, 100 ul of seminal plasma were removed and combined with 900 ul of 25% acetonitrile (ACN) containing 0.1% (w/v) trifluoroacetic acid (TFA) for separation over a 3 ml reversed phase C_4 extraction column (Vydac™, Hesperia, CA). Prior to loading samples, columns were equilibrated using 500 ul of 100% ACN, followed by 500 ul of 5% ACN with 0.1% (w/v) TFA. After equilibration, samples were loaded and allowed to pass through via gravity flow and positive pressure. Columns were washed one time using one ml of 5% ACN with 0.1% (w/v) TFA to remove weakly bound components. The bound fraction containing FAA

was then eluted from the column by applying one ml of 60% ACN with 0.1% (w/v) TFA. Eluted fractions were collected and dried in a SC100 SpeedVac (Savant Instruments, Inc., Farmingdale, NY) and stored at -80^{o} C. The aforementioned separation procedures for FAA were adapted from the isolation and purification of FAA (McCauley et al., 1999).

Assay plates (Pro-Bind™, Becton Dickinson Labware, Lincoln Park, NJ) were coated with anti-recombinant FAA (rFAA) antiserum (McCauley et al., 2004) which was diluted 1:1000 in capture buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate; pH=9.6). Diluted antiserum (100 ul) was added to each well and incubated overnight at 4^{o} C. After the overnight incubation, wells were rinsed six times with wash buffer (20 mM sodium phosphate, 1.5 mM potassium phosphate, 134 mM sodium chloride, 2.7 mM potassium chloride and 0.05% Tween 20; pH=7.2). All wells were blocked using 200 ul of capture buffer containing 1.15% non-fat dry milk and incubated while shaking at 37^{o} C for one h. The plates were again rinsed six times. Eluted, dried protein was resuspended using 200 ul of wash buffer and 100 ul applied per well. Dilutions of purified, recombinant FAA in wash buffer served as the reference standard for the assay. All wells were performed in duplicate. Plates were incubated at 37^{o} C for one h while gently shaking. Plates were then rinsed six times and 100 ul of rFAA-horseradish peroxidase (HRP) conjugated antibody diluted 1:250 in wash buffer was applied to each well. Plates were again placed into a shaking incubator at 37^{o} C for one h. After the final incubation, plates were washed six times and developed using 100 ul of 3,3',5,5' tetramethylbenzidine (TMB; 1-Step™ Ultra TMB-ELISA, Pierce). Development was allowed to proceed for 30 min at room temperature and the reaction stopped by the

addition of 100 ul of 2 M sulfuric acid (H_2SO_4) to each well. Absorbance was measured at 450 nm using a 96-well Multiskan Ascent plate reader (Labsystems; Vienna, VA).

4. Enzyme-linked Immunosorbent Assay (ELISA) for Tissue Inhibitor of Metalloproteinases-2 (TIMP-2)

The concentration of TIMP-2 in bovine seminal plasma from 35 bulls was determined using a commercially available human TIMP-2 ELISA system (Biotrak; Amersham Biosciences, Piscataway, NJ). This system had previously been validated and used to detect TIMP-2 in serum from mouse, rat, guinea pig, rabbit and bovine (Fujimoto et al., 1995). The assay is based on a two-site ELISA sandwich format using two monoclonal antibodies specific to the amino and carboxyl terminus of TIMP-2. As described for blood plasma (Lein et al., 1997) and human seminal plasma (Baumgart et al., 2002b) samples, bovine seminal plasma was diluted using 50 mM phosphate-buffered saline (pH= 7.5) containing 1% bovine serum albumin (BSA) to measure the samples in the working range of the TIMP-2 assay. All standards and samples were assayed in duplicate. Incubation and wash procedures were carried out according to manufacturer's directions using supplied Biotrak reagents. After the addition of the TMB substrate, the reaction was allowed to proceed for 30 min. The reaction was stopped using 100 ul of 2 M H_2SO_4. Absorbance was measured at 450 nm using a 96-well Multiskan Ascent plate reader.

D. Statistical Analysis

A linear model was fit to the individual standard curves for the FAA and TIMP-2 ELISAs. The resulting linear curve and equation for each ELISA was used to determine the concentration of FAA and TIMP-2 in bovine seminal plasma samples.

The fertility of sires was assessed by using the total number of bull calves produced by each sire during the three-year fertility trial since only male calves were examined for paternal origin. Models were fit to the three dependent variable data sets of bull calves sired, concentration of FAA and concentration of TIMP-2. Age was considered a fixed effect in all three models. Data were analyzed using PROC MIXED of the Statistical Analysis System (SAS, 1999) and least squares means (lsmean) were computed for the response variables. An alpha level of $P \leq 0.05$ was used to control for Type I errors.

The model for the analysis of bull calves sired, FAA and TIMP-2 concentrations was:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where,

Y_{ij} = number of bull calves, or concentration of FAA, or concentration of TIMP-2

μ = population mean

α_i = i^{th} age effect (1, 2, 3 years of age)

ε_{ij} = error term

In addition, correlation analyses were also performed to determine the relationship of FAA and TIMP-2 concentrations to the number of bull calves sired, and to

determine if those fertility-related protein concentrations in seminal plasma were inter-related.

E. Results

1. Quantification of Fertility-associated Antigen (FAA) in Bovine Seminal Plasma by ELISA

The concentration of FAA in bovine seminal fluid was analyzed from 46 individual bulls. Recombinant FAA was used to develop a standard curve (Figure 3.1) that ranged from 0 to 3500 ng/ml. A linear line was fit (R^2=0.969) to the standards to predict the concentration of FAA in unknown samples. The concentration of FAA ranged from 0 to 37.9 ug FAA/ml of seminal plasma (Table 3.1). The overall lsmean±se for 46 bulls was 6.66±1.48 ug FAA/ml of seminal plasma (Table 3.1). The concentration of FAA in seminal plasma was not affected (P>0.70) by bull age (Figure 3.2). However, there was a significant (P<0.0001) age effect for the number of calves sired by each age group (Figure 3.3). There was no difference (P>0.10) in the total number of bull calves sired by bulls one year or less than one-year of age. Two-year old bulls tended (P<0.07) to sire more calves than one-year old bulls. Three-year old bulls sired significantly (P<0.01) more calves than any other age group. Overall, the concentration of FAA in seminal plasma, measured by the FAA ELISA, was not reflective of bull fertility potential as there was no correlation (R^2 = 0.04) between the number of bull calves sired and the concentration of FAA in seminal plasma. However, the use of older bulls in multi-sire pastures was beneficial.

2. Quantification of Tissue Inhibitor of Metalloproteinases-2 (TIMP-2) in Bovine Seminal Plasma by ELISA

The Biotrak TIMP-2 ELISA system was used to determine the concentration of TIMP-2 in bovine seminal plasma. This system had previously been used to detect TIMP-2 in serum from mouse, rat, guinea pig, rabbit and bovine (Fujimoto et al., 1995). Figure 3.4 represents the standard curve for the TIMP-2 assay which ranged from 0 to 128 ng/ml. A linear line was fit (R^2=0.994) to the standards to predict the concentration of TIMP-2 in unknown samples (Figure 3.4). The concentration of TIMP-2 ranged from 0.92 to 2.41 mg of TIMP-2/ml of seminal plasma (Table 3.1). The lsmean±se concentration of TIMP-2 in bovine seminal fluid for 35 bulls was 1.18±0.045 mg/ml (Table 3.1). As the age of bulls increased, there was a tendency (P<0.10) for an increase in the concentration of seminal TIMP-2 (Figure 3.5). There was no difference (P>0.40) in the concentration of TIMP-2 in seminal fluid from bulls less than two-years of age. TIMP-2 concentrations were similar (P>0.10) for two and three-year old bulls; however, three-year old bulls produced significantly (P<0.03) more TIMP-2 in seminal plasma than one-year old bulls. Also, there was also a tendency (P<0.08) for three-year old bulls to have a higher concentration of TIMP-2 in seminal plasma versus bulls under one-year of age. The overall effect of age was P=0.09 when the dependent variable, TIMP-2 concentration, was analyzed. The concentrations of TIMP-2 in relationship to the number of bull calves sired was weakly correlated (R^2=0.14).

In relationship to age of bull and fertility, there was a significant (P<0.0001) age effect in the number of calves sired (Figure 3.6). Bulls that were <1, 1, 2 and 3-years of

age sired on average 3.75, 17.3, 35.7 and 56 total bull calves, respectively. The overall standard error across all ages for the number of bull calves sired was ±6.55. Three-year old bulls sired a significantly ($P<0.02$) greater number of calves than any other age group. Two-year old bulls sired more ($P<0.03$) calves than bulls one-year or less in age. However, there was no difference ($P>0.20$) in the number of calves sired by bulls one-year or less than one-year in age.

Older bulls appeared to have greater concentrations of TIMP-2 in seminal plasma (see Figure 3.5). This trend appeared to also be reflected in the increasing number of calves sired by older bulls. Therefore, higher concentrations of TIMP-2 in bovine seminal plasma may be an indicator of bull fertility.

F. Discussion

An ELISA was developed to quantify FAA in bovine semen using polyclonal antibodies directed against recombinant FAA. The anti-recombinant FAA antibody has previously been shown to be mono-specificfor FAA (McCauley et al., 2004). FAA could not be quantified in seminal plasma by ELISA until it was separated based on biochemical properties using methods previously described by McCauley et al. (1999). That fractionation allowed quantification of FAA by ELISA following the removal of inhibitory factor(s) present in bovine seminal plasma.

The concentration of FAA in bovine seminal plasma ranged from 0 to 37.9 ug/ml. FAA was initially separated from bovine seminal fluid by Miller et al. (1990), and it was later purified and biochemically identified using seminal plasma from one vasectomized bull (McCauley et al., 1999). The bull that was vasectomized was chosen as the original

FAA donor due to the presence of high concentrations of FAA visualized by Western blots of FAA fractionated from his semen. From those data, the concentration of FAA in seminal plasma was calculated to be approximately 20 ug/ml (unpublished data). These results agree with that estimation, but the concentration across a larger population of bulls was lower (6.66±1.48 ug/ml). The molecular weight of the recombinant FAA standard reflects two-thirds (70.9%) of the native molecular weight of FAA, 22,000 Daltons versus 31,018 Daltons. Therefore, the concentration of FAA based on an adjusted molecular weight ratio would be less, 4.72±1.05 ug/ml. Nevertheless, those concentrations do not take into account the quantity of FAA that may be sperm-associated. An attempt was not made to ascertain the bound fraction of FAA to sperm, only to develop a new procedure that can be used to accurately assess concentrations of FAA in bovine seminal plasma.

The results from this study indicated the concentration of FAA in bovine seminal fluid was not reflective of bull fertility potential since there was no relationship between concentrations of FAA measured by ELISA and the number of calves sired by the 46 bulls used in this experiment. This finding validates the importance of assessing sperm-bound FAA, and not unbound or free FAA when selecting replacement sires for increased fertility. Although the presence of FAA in seminal plasma is important for sperm exposure to this high-affinity heparin-binding protein, the binding ability and amount of FAA actually bound to sperm may be more indicative of bull fertility. It is possible that the lower concentrations observed in seminal fluid is due, in part, to the amount of FAA that bound to sperm at ejaculation. All previous studies showing a relationship between

FAA and fertility utilized Western blots of sperm-associated extracts of proteins (Bellin et al., 1996; 1998; and Sprott et al., 2000).

Sperm from high fertility bulls exhibit a greater response to heparin-induced capacitation than sperm from low fertility bulls (Ax et al., 1985). The presence of FAA on sperm from those higher fertility bulls is proposed to mediate that interaction. The lack of a relationship to fertility in this study may be due to assessing the concentration of FAA in seminal plasma and not sperm. Therefore, the concentration of FAA in seminal plasma alone would not offer predictive value for fertility.

A commercially available TIMP-2 ELISA was also used to determine the concentration of TIMP-2 in bovine seminal fluid. This assay has been used to determine the concentration of TIMP-2 in serum from a number of species (Fujimoto et al., 1995) and in human seminal plasma (Baumgart et al., 2002a and 2002b). The monoclonal antibodies used in the TIMP-2 ELISA were developed from synthetic oligopeptides specific to the amino and carboxyl terminus of TIMP-2 (Fujimoto et al., 1993) based on the amino acid sequence first determined by Stetler-Stevenson et al. (1989).

TIMP-2 is considered a major bovine seminal plasma protein (Calvete et al., 1996). That study isolated bovine TIMP-2 in seminal fluid by heparin-Sepharose chromatography and reported 52±20 mg of TIMP 2 per 100 ml of seminal fluid. However, that concentration was calculated after size-exclusion and affinity chromatography procedures that could have resulted in a lower recovery of TIMP-2 from bovine seminal plasma. Furthermore, concentrations of TIMP-2 were assayed in pooled seminal plasma samples which did not allow for male-to-male variation reported herein.

Data obtained in this study indicated approximately a two-fold greater concentration of TIMP-2 in bovine seminal plasma than originally reported by Calvete et al. (1996). Both studies confirm that bovine seminal plasma media is a highly enriched source of TIMP-2; however, the biological significance of this fact remains to be elucidated.

The matrix metalloproteinases and tissue inhibitor of metalloproteinases are mediators of reproductive function in males and females (reviewed by Hulboy et al., 1997). Therefore, monitoring concentrations of TIMP-2 may be a valuable tool for assessing male accessory gland function. The concentration of TIMP-2 in human seminal plasma has previously been established by Baumgart et al. (2002a and 2002b). The mean concentration of TIMP-2 in human seminal plasma ranged from 4.44±2.65 to 5.95±3.63 ug/ml for normozoospermic and azoospermic patients, respectively. The concentration of human seminal TIMP-2 was nearly 200-fold less than the concentration of TIMP-2 detected in the present study. Although those data were limited by the number of human patients, no correlation was found between TIMP-2 concentrations and sperm motility, morphology or concentration of sperm. Thus, TIMP-2 concentrations in human seminal plasma do not appear to be reflective of fertility potential, but the importance related to overall reproductive tract function or health remains to be determined. It is plausible that accessory sex glands of bulls undergo greater gland remodeling and reconstruction due to their larger secretory nature, thus higher concentrations of TIMP-2 are present to regulate this activity. If the latter were true, bulls which possess a greater concentration of TIMP-2 in seminal plasma could imply a healthier reproductive tract, and that could be indicative of higher fertility in those males. The data herein suggested

that bulls which produced seminal plasma with higher concentrations of TIMP-2 also tended to produce more calves.

Previous research has identified that FAA (Bellin et al., 1994, 1996, 1998; Sprott et al., 2000) and TIMP-2 (Dawson et al., 2002) can be used to predict bull fertility. Those studies used the presence or absence of FAA/TIMP-2 in sperm membrane extracts only with detection by Western blots. Therefore, fertility outcomes in this study cannot be directly compared to previous studies. However, it can be concluded that concentrations of FAA in seminal plasma do not appear to change as bulls become older, and TIMP-2 concentrations are higher compared to other species. Although there was no correlation (R=0.10) between the concentrations of FAA and TIMP-2 in seminal plasma, the production of heparin-binding proteins, which include FAA and TIMP-2, is dependent on testosterone (Nass et al., 1990). The FAA and TIMP-2 ELISAs may serve as useful assays to screen for those proteins in relationship to circulating androgen concentrations to determine the duration of androgen exposure necessary for production of FAA and TIMP-2.

In conclusion, an enzyme-linked immunosorbent assay to measure the concentration of FAA in seminal plasma was developed and a commercially available ELISA for TIMP-2 was utilized to quantify TIMP-2 without accounting for FAA or TIMP-2 that may be bound to sperm. Future studies should employ the FAA and TIMP-2 ELISAs to quantify concentrations of those proteins in sperm-only and seminal plasma fractions from the same individuals. These methods are less cumbersome and can be

used to replace the traditional immunoblotting technique that has been used in previous studies relating FAA and TIMP-2 to fertility potential of bulls.

Acknowledgements

The authors would like to acknowledge Dr. Jerry McVicker (Midland Bioproducts, Boone, IA) for the HRP-conjugation of anti-rFAA antibody and for his assistance in developing the FAA ELISA.

Figure 3.1. Titration of recombinant fertility-associated antigen (FAA) standard curve. All dilutions were performed in duplicate using 0, 10, 100, 500, 1000, 2000 and 3500 ng/ml of purified recombinant FAA. Absorbance was measured at 450 nm. A linear equation was used to determine the concentration of FAA in unknown bovine seminal plasma samples.

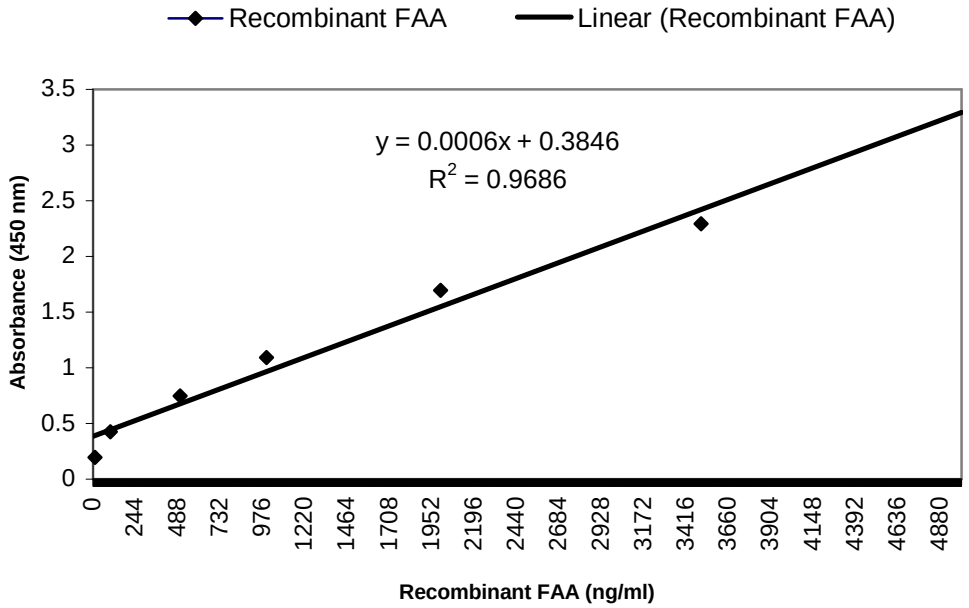
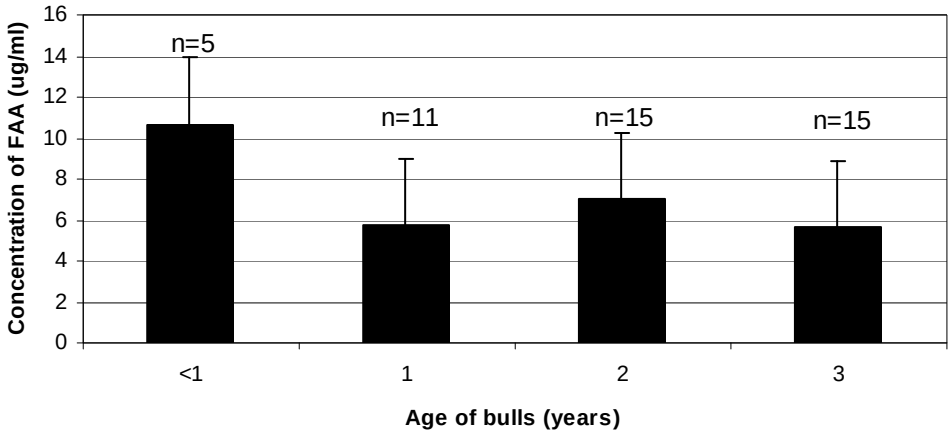


Figure 3.2. The least squares mean (lsmean±se) concentration (ug/ml) of FAA in bovine seminal plasma with respect to bull age (years)*. FAA concentrations in seminal plasma were determined from 46 separate bulls using the FAA ELISA. The number (n) of bulls in each age group is shown.



* No significant age effect (P>0.70).

Figure 3.3. The least squares mean (lsmean±se) of total bull calves produced in relationship to sire age (years)* for the 46 bulls screened with the FAA ELISA. All bull calves were verified by parental determination using DNA-based genotyping.

[a,b,c] Means with different superscripts differ (P<0.05).

* Significant (P<0.0001) age effect.

Figure 3.4. Tissue inhibitor of metalloproteinases-2 (TIMP-2) standard curve using the Biotrak TIMP-2 ELISA system. The standard curve was developed using 0, 8, 16, 32, 64 and 128 ng/ml of TIMP-2. Absorbance was measured at 450 nm. A linear equation was used to determine the concentration of TIMP-2 in unknown bovine seminal plasma samples.

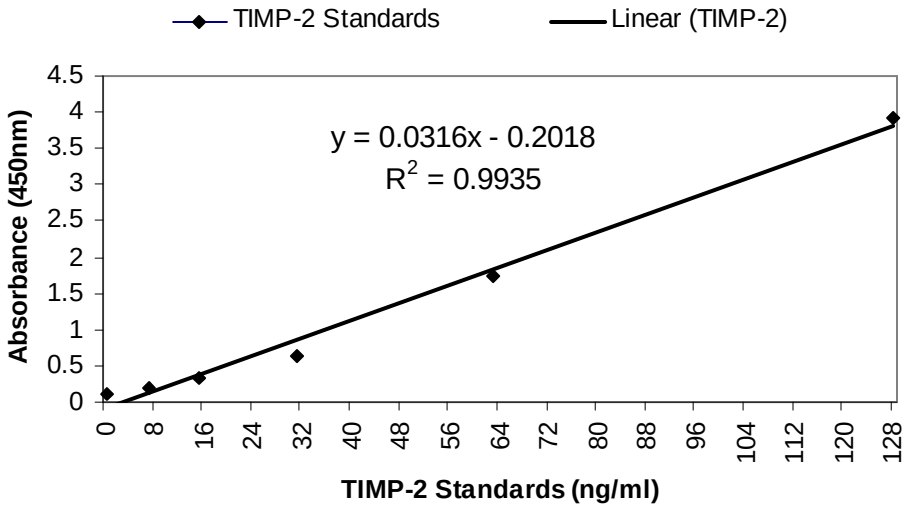
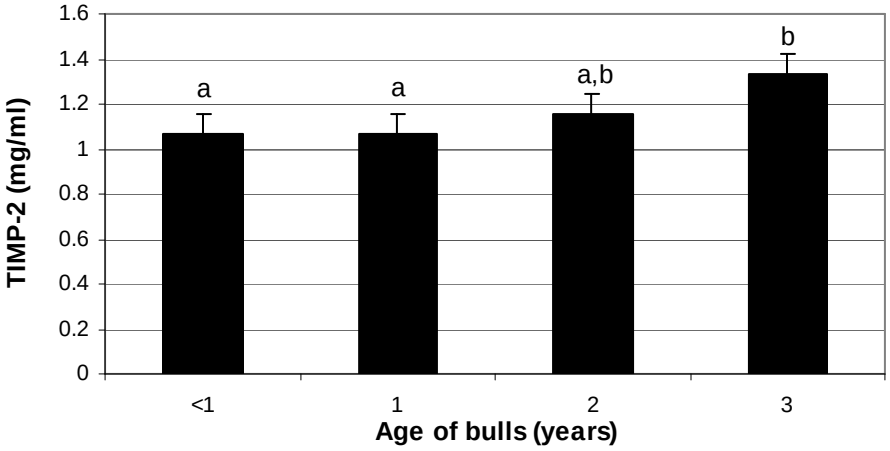
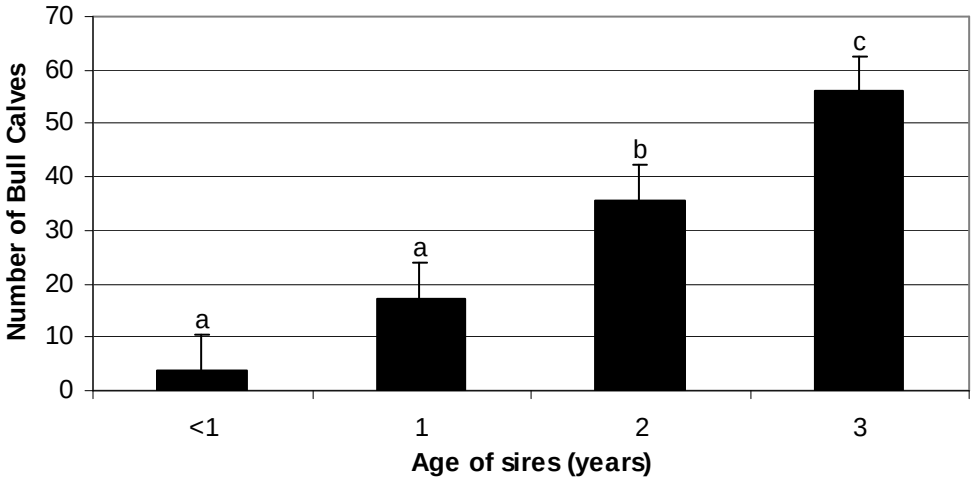


Figure 3.5. The least squares mean (lsmean±se) concentration (mg/ml) of TIMP-2 in bovine seminal plasma with respect to bull age (years)*. TIMP-2 concentrations in seminal plasma were determined for 35 bulls using the Biotrak TIMP-2 ELISA system.



[a,b] Age 1 versus age 3 was significant (P<0.05).
Age <1 versus age 3 (P<0.08).
* Overall effect of age (P<0.10).

Figure 3.6. The least squares mean (lsmean±se) of total bull calves produced in relationship to sire age (years)* for the 35 bulls used in the TIMP-2 ELISA experiment. All bull calves were verified by parental determination using DNA-based genotyping.



[a,b,c] Means with different superscripts differ (P<0.05).
* Significant (P<0.0001) age effect.

Table 3.1. Overall distribution of the concentration[a] of fertility-associated antigen (FAA)[a] and the concentration[b] of tissue inhibitor of metalloproteinases-2 (TIMP-2) in bovine seminal fluid quantified by ELISA.

	Number of Bulls	Minimum	Maximum	Median	Mean±SE	95% Confidence Interval
FAA	46	0	37.94	0.19	6.66±1.48	±2.9
TIMP-2	35	0.92	2.41	1.09	1.18±0.045	±0.09

[a] Concentration of FAA ug/ml.
[b] Concentration of TIMP-2 mg/ml.

CHAPTER FOUR

I. A Novel 700 bp Bovine Genomic DNA Marker for Fertility Potential of Bulls[a]

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A. Abstract

All advanced diagnostic markers for bull fertility currently rely on immuno-based detection methods. Four proteins have been isolated and characterized from bull semen that can be used to predict fertility of bulls. The presence of a 24 kDa heparin-binding protein in bovine semen, identified as tissue inhibitor of metalloproteinases-2 (TIMP-2), corresponded to a 13% increase in fertility over herdmates that were TIMP-2 negative. To screen for the TIMP-2 protein in semen, bulls must be artificially collected which generally requires bulls to be greater than 10 months of age. In contrast, a genomic DNA (gDNA) marker for the TIMP-2 gene could be applicable to an embryo or using blood or tissue sample from a neonatal calf.

Using TIMP-2 cDNA gene specific primers and amplified fragment length polymorphism (AFLP) methodology, bovine gDNA isolated from sperm was amplified to screen for a unique 700 bp genomic marker that is related to bull fertility. Preliminary data had indicated the absence of this gDNA product corresponded to bull sterility. Results indicated that bulls which were negative for the 700 bp marker were three-times more likely to not sire a calf in a given year. That likelihood was statistically significant by Chi-square analysis. In addition, the amplified 700 bp product was cloned and sequenced. Interestingly, no homology was observed between the amplified product and the bovine TIMP-2 gene. If future studies continue to advance this DNA fingerprint analysis, this novel genetic marker may prove to be a useful tool to predict bovine fertility since it lends itself for use in both males and females.

B. Introduction

Fertility is five to 10 times more important to livestock producers than any other measurable performance trait (Trenkle and Wilham, 1977). Objective methods to select future sires based on fertility are primarily limited to protein or antibody-based detection methods. Four proteins have been identified in bovine semen that are related to bull fertility potential. Those proteins are osteopontin (OPN; Cancel et al., 1997; 1999), lipocalin-Type D prostaglandin synthase (PGD; Gerena et al., 1998; 2000), Fertility-associated antigen (FAA; Bellin et al., 1998; McCauley et al., 1999), and Type-2 tissue inhibitor of metalloproteinases (TIMP-2; McCauley et al., 2001). A multiple regression analysis using the presence of OPN and PGD in bovine semen was significantly ($R = 0.89$) related to bull fertility (Killian et al., 1993). A series of field trials conducted by Bellin and coworkers (1994, 1996 and 1998) used the presence (or absence) of FAA on sperm to segregate bulls, and FAA positive bulls were 19% more fertile than FAA negative bulls. TIMP-2 has also been used to determine the fertility of bulls and TIMP-2 positive bulls were 13% more fertile than their TIMP-2 negative herdmates (Dawson et al., 2002). Production of the latter two heparin-binding proteins was testosterone-dependent (Nass et al., 1990) which indicated that puberty must be reached prior to appearance of FAA and TIMP-2 as seminal constituents.

All of those aforementioned proteins also require semen to be collected which dictates the use of peri-pubertal bulls. Therefore, protein and antibody-based fertility diagnostic procedures are only applicable after there is a substantial financial investment into those males. DNA-based fertility markers would allow for selection pressure on

fertility in both genders at a day of age. Over 200 genetic defects have been associated with infertility or sub-fertility in other animal models (reviewed by Matzuk and Lamb, 2002). Those results confirm the complexity of defining fertility markers that are indicators of fertility. However, since the previously mentioned proteins are related to bull fertility, they may serve as candidate genetic markers for fertility prediction in cattle.

DNA fingerprinting by amplified fragment length polymorphism (AFLP) methodology is a molecular technique that allows for the visualization of differences in DNA sequences between individuals (Vos et al., 1995). It also allows for the identification of DNA markers for a particular trait. The outcome is assessed by the presence or absence of an amplified nucleotide sequence. In addition to the clear outcome, AFLP's have a high degree of accuracy and reproducibility (Jones et al., 1998; Matthes et al., 1998). Furthermore, PCR platforms can be multiplexed to allow for the screening of numerous AFLPs at one time with primers designed around multiple genes of interest. Although the AFLP approach to identify genetic markers has numerous advantages, their implementation into livestock systems has been primarily limited to cattle (Ajmone-Marsan et al., 1997; Buntjer et al., 2002; Tsuji et al., 2004) and pigs (Plastow et al., 1998; Thurston et al., 2002; Cameron et al., 2003).

The majority of reports utilizing AFLP methods are for the construction of phylogenic trees; however, Thurston and coworkers (2002) assessed AFLP markers linked to genes controlling boar sperm viability. Sixteen candidate genetic markers were identified by AFLP analyses that were reflective of boar semen freezing quality. Likewise, others have performed AFLP marker assisted selection for high merit marbling

characteristics in cattle (Tsuji et al., 2004). Results from the latter study suggested that AFLP fingerprinting may be used effectively to develop animal breeding schemes without identification of all the genes that affect the ultimate trait of interest.

Therefore, our objective was to use AFLP methodology with gene-specific TIMP-2 primers and develop a bovine DNA fingerprint analysis. Bovine TIMP-2 specific primers were chosen based on the TIMP-2 relationship to bull fertility. Preliminary data using the TIMP-2 primers on a limited population of sires (N=13) had indicated the fingerprint analysis was a predictor of bull sterility when a 700 bp PCR band was absent (H.M. Zhang, personal communication). In addition, the 700 bp product was cloned and sequenced to gain further information regarding this potential bovine gDNA fertility marker. The AFLP product outcome for 55 bulls and the sterility/fertility of those individuals based upon parentage determination of the resulting offspring are presented herein.

C. Materials and Methods

All chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated.

1. Fertility Trial

A fertility trial was conducted over three consecutive breeding seasons using only bulls that were qualified as potential satisfactory sires following a breeding soundness exam. All bulls were maintained at a constant ratio of one bull per 25 cows for a 60-d breeding season in randomly assigned multi-sire pastures. Blood samples were taken on all potential sires and all of the resulting male offspring (one-half of the calf crop). For

each potential sire, it was assumed there was a 50% chance to sire a male calf and 50% chance to sire a female calf, thus, limiting the number of calves utilized for verification of parentage. All parental testing was performed on a contractual basis by Maxaam Analytics, Inc. (Guelph, Canada). A total of 15 microsatellite markers was used for parentage determination.

Semen collected during the breeding soundness exam was stored at –20^{o} C until gDNA isolation.

2. Isolation of Bovine Genomic DNA (gDNA)

Bovine gDNA was isolated from sperm collected from 55 Angus bulls. Neat semen from each individual bull was then thawed on ice, vortexed briefly and 250 ul were pipetted and placed into a 1.5 ml microcentrifuge tube with one ml of sterile dd H_2O. Samples were centrifuged at 600 x g for 5 min, and the supernatant was discarded. The sperm pellets were resuspended with sterile dd H_2O with the wash step repeated an additional three times. After the final wash, sperm pellets were resuspended in digestion buffer containing 0.01 M Tris-hydrochloride (Tris-HCl; pH=8.0), 0.05 M sodium chloride (NaCl), 0.01 M ethylenedinitrilo-tetraacetic acid (EDTA), 2 % sodium-dodecyl sulfate (SDS), 0.025 M dithiothreitol (DTT) and 200 ug/ml proteinase K. Samples were incubated for three hours at 60^{o} C while being gently mixed in an Eppendorf Thermomixer 5436 (Brinkmann Instruments, Westbury, NY). One-third volume of 5 M sterile-filtered NaCl was added at the end of the incubation and samples were allowed to equilibrate at room temperature (RT) for 5 min. All samples were then centrifuged at 10,000 x g for 5 min at RT. Supernatants were aspirated and placed into separate 5 ml

sterile culture tubes. Cold-ethanol (100%) was added at approximately two to three times the aspirate volume to precipitate DNA. The precipitated DNA was recovered by using a sterile glass rod. Recovered DNA was washed twice using 70 % cold-ethanol and the DNA/glass rod was placed into a new 1.5 ml microcentrifuge tube. Following DNA extraction, all tubes were placed into an evacuated desiccator and dried overnight. Dried DNA was resuspended with sterile dd H_2O. DNA concentration was determined using a spectrophotometer (GeneQuant II; Pharmacia Biotech, Cambridge, England) and visualized by agarose gel electrophoresis prior to PCR.

3. Amplification of gDNA Products by Polymerase-chain Reaction (PCR)

Fifty ng of gDNA template isolated from sperm from each respective bull was used separately in a 25 ul PCR reaction consisting of 10mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton® X-100, 2.0 mM $MgCl_2$, 200 µM each of dATP, dTTP, dCTP and dGTP and 0.3 units of *Taq* DNA polymerase per reaction (all obtained from Promega Corporation, Madison, WI) in conjunction with 800 nM of each forward 5'-GCGTTCTGCCTCCTGCTGCTG-3' and reverse 5'-CGCTTGATGGGGTTGCCGTAG-3' primer in a 200 µl thin wall PCR tube (Bio-Rad, Hercules, CA). PCR cycling conditions were 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C for 35 cycles with a 10 min final extension at 72^o C carried out in a Peltier Thermal Cycler 200 (PTC-200; MJ Research, Inc, Watertown, MA). The PCR products were analyzed directly by electrophoresis in an ethidium bromide-stained 2% agarose, 90 mM Tris, 90 mM boric acid and 2 mM EDTA (TBE buffer, pH=8.3) gel to determine the DNA pattern for each bull. A 100 bp PCR DNA ladder (EZ Load 100 bp Molecular Ruler, Bio-Rad) served as

reference standard. Gel images were captured using an ultraviolet light box and CCD camera linked to Alpha Imager™ software (Alpha Innotech Corporation, San Leandro, CA).

4. Cloning and Sequencing of PCR Products

The PCR conditions described above amplified a series of DNA products ranging from approximately 300 bp to 700 bp in size (Figure 4.1). Preliminary experiments indicated that absence of the 700 bp band was related to male sterility. Therefore, the 700 bp band was cloned and sequenced to determine the amplified region. Fresh PCR products were gel extracted by excising the band of interest using the QIAEX II gel extraction kit (Qiagen Inc., Valencia, CA) per manufacturer's directions. Each eluted PCR product was directly cloned into the pCR®4-TOPO® vector supplied with the TOPO® TA PCR cloning kit (Invitrogen, Carlsbad, CA) following manufacturer's recommendations. Cloned products were transformed into TOP10 One Shot® (Invitrogen) chemically competent bacterial cells and plated onto pre-warmed selective Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 1.5% agarose, pH=7.0) plates supplemented with 50 ug/ml of kanamycin antibiotic. Two volumes were plated to ensure an adequate number of well-spaced colonies for selection purposes. Plates were incubated at 37^{o} C overnight.

Multiple colonies from each transformation were then selected and cultured in LB media (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH=7.0) with kanamycin (50 ug/ml) for an additional 12 h using a shaking (225 rpm) 37^{o} C incubator (Environ; Barnstead International, Dubuque, IA). Following propagation, bacterial cells were

harvested by centrifugation at 1,000xg for 10 min at RT. Cells were lysed and plasmids purified using the Qiagen Spin Miniprep columns (Qiagen, Valencia, CA). Re-PCR was performed to validate the cloned insert prior to sequencing using 10 ng of purified plasmid DNA, vector supplied primers and PCR Master Mix (Promega Corporation, Madison, WI). The thermocycler conditions were 94°C, 55°C and 72°C for one minute each for 35 cycles. PCR products were visualized as previously described for agarose gel electrophoresis. Plasmids that maintained a discrete single PCR band of the correct molecular size after PCR were then sent to the University of Arizona DNA sequencing facility. All samples were sequenced using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis and comparisons were performed using The Biology WorkBench version 3.2 software available at *http://workbench.sdsc.edu/*. Homogeneity to other known DNA sequences was performed using nucleotide-nucleotide blast at the National Center for Biotechnology (NCBI; *http://www.ncbi.gov*) and The Institute for Genomic Research (TIGR; *http://www.tigr.org/*).

D. Statistical Analysis

Fertility data were analyzed for bulls (N=48) that had three consecutive breeding seasons using PROC MIXED in Statistical Analysis System (SAS, 1999). The model was fit to the dependent variable number of bull calves sired to assess sire fertility. The full model included the fixed effects of age, calf crop and AFLP status (presence or absence of 700 bp band), and the age by AFLP interaction. Calf crops (i.e. breeding seasons one, two and three) within bull were considered repeated measures. Least

squares means were computed for all fixed effects. An alpha $P \leq 0.05$ level threshold was used to determine significance in order to control for Type I errors.

The model for the analysis of number of calves was:

$$Y_{ijkl} = \mu + a_i + b_j + c_k + (ac)_{ik} + \varepsilon_{ijkl}$$

where,

Y_{ijkl} = number of bull calves sired

μ = population mean

a_i = i^{th} age effect (<1, 1, 2, 3, 4, 5)

b_j = j^{th} calf crop effect (calf crop 1, 2, 3)

c_k = k^{th} AFLP effect (positive or negative for 700 bp PCR band)

$(ac)_{ik}$ = interaction between i^{th} age and k^{th} AFLP effect

ε_{ijk} = error term

Overall reproductive performance of the bulls was also evaluated by determining the success of siring or not siring a calf in a given year. This computation was based upon preliminary data that suggested the absence of a 700 bp band was a predictor of bull sterility. A successful event (i.e., calf produced) was numerically assigned a one and an unsuccessful event (i.e., no calf produced) assigned a zero for all bulls in a given year. Therefore, for each bull, a total of three events could have occurred over the fertility trial and those events were deemed successful or unsuccessful for each given year or breeding season. For both the total number of successful and unsuccessful events, probability

estimates were obtained from a logistic regression model based on the potential to sire at least one calf in a given year or the potential for no calf in a given year (sterile year).

The event data described above were analyzed using PROC MIXED. Event was used as the dependent variable and the fixed effects were age of bull, AFLP status, and year (calf crop). Least squares means were computed for the AFLP response variable.

The model for the event analyses was:

$$Y_{ijklm} = \mu + a_i + b_j + c_k + d_l + (bd)_{jl} + \varepsilon_{ijklm}$$

where,

Y_{ijklm} = event (calf sired or no calf sired in a breeding year)

μ = population mean

a_i = i^{th} age effect (<1, 1, 2, 3, 4, 5)

b_j = j^{th} AFLP effect (positive or negative for 700 bp PCR band)

c_k = k^{th} year effect (breeding year 1, 2, 3)

d_l = random bull effect ~$N(0, \sigma^2_{bull} I)$

$(bd)_{jl}$ = random interaction between AFLP and bull ~$N(0, \sigma^2_{AFLP/bull} I)$

ε_{ijklm} = error term

E. Results

1. PCR Amplification of the 700 bp Bovine gDNA Product

Using the forward 5'-GCGTTCTGCCTCCTGCTGCTG-3' and reverse 5'-CGCTTGATGGGGTTGCCGTAG-3' primers to screen bovine gDNA isolated from

sperm resulted in the successful amplification of a series of PCR products ranging in size from approximately 300 to 700 bp (Figure 4.1). In Figure 4.1, the 700 bp product was clearly absent in some bulls (Lanes 3 and 4). Overall, gDNA from 55 bulls were subjected to the DNA fingerprint analysis. Figure 4.2 represents the total number of bulls that were positive and negative for the 700 bp product and that failed amplification (absent of any PCR products) under the defined PCR conditions. After four separate attempts, no PCR products were amplified for two of the 55 bulls. The overall success of the PCR DNA fingerprint analysis for all bulls was 96% (53/55). There were 12/53 (22.6%) bulls which were negative for the 700 bp product.

2. Relationship to Bull Fertility

For bulls that were positive or negative for the 700 bp band, there was no difference ($P=0.45$) in the mean±SE number of calves produced, 9.7±1.17 vs. 8.27±1.08, respectively (Figure 4.3). However, there was a significant ($P<.0001$) age effect (Figure 4.4). Bulls greater than four years of age produced significantly ($P<0.05$) more calves than younger bulls (less than three years of age). Although age was significant in the model, there was no age by AFLP (700 bp present or absent) interaction ($P>0.70$; Figure 4.5), consistent with this being a genomic marker. Therefore, the increase in the number of calves sired was directly related to increased age of bulls.

The absence of the amplified 700 bp product was originally associated with sterility of an Angus bull (H.M. Zhang, personal communication). Therefore, we computed the number of events that were successful (at least one bull calf sired by a bull) and unsuccessful (no calf sired) over three consecutive years for bulls categorized as

AFLP negative or AFLP positive (Table 4.1). There was a three-fold increase in the likelihood (33%) of a bull that was AFLP negative for the 700 bp band to have a sterile year compared to herdmates that were AFLP positive (11%). That difference was statistically significant by Chi-square analysis ($P<0.01$). The overall reproductive performance of the Angus bulls is presented in Table 4.1. As previously indicated, only male calves underwent parentage determination. The total number of bull calves born per breeding opportunity was similar ($P>0.05$); however, the opportunity to not sire a calf (i.e. sterile year) was numerically higher when those bulls were AFLP negative. The aforementioned data were strictly computed based on the findings of preliminary data and the absence of the 700 bp marker's association with bull sterility.

3. Cloning and Sequence Analysis of the 700 bp Product

TIMP-2 gene specific primers were used to amplify the 700 bp gDNA product. Those primers amplified a 175 bp TIMP-2 product from accessory sex gland cDNA (McCauley et al., 2001). That product is illustrated in Figure 4.1, lane 8 (PCR control). Figure 4.6 compares the nucleotide sequences obtained from the 700 bp products of two separate Angus bulls. Sequence analysis indicated only a 50% homology between the two sequences. No homology was observed to the bovine TIMP-2 gene except for primer sequences. Both sequences appeared to be unique, as nucleotide-nucleotide blast search at NCBI identified no significant matches over 40 bp with the majority consisting of short 15-20 bp matches. Using the TIGR bovine database, 206/212 nucleotides spanning bp regions 450 through 661 of the cloned sequences were 97% identical to a portion of the bovine embryonic and extra-embryonic library (reference TC290499).

F. Discussion

Bull DNA-based fertility diagnostics offer many advantages over currently available semen or protein/antibody-based procedures. Selection pressure on fertility could be placed at a day of age or at weaning prior to substantial economic investment into those males. Moreover, DNA diagnostics would be applicable to both genders.

DNA genotypic analyses are becoming widely used to evaluate genotypes in cattle (Ajmone-Marsan et al., 1997; Buntjer et al., 2002) and pigs (Plastow et al., 1998; Cameron et al., 2003). Others have utilized the AFLP approach to identify markers that may indicate cryopreservation capacity of boar sperm (Thurston et al., 2002) or for desirable carcass characteristics in cattle (Tsuji et al., 2004). Those studies have identified DNA patterns that may be used as predictors for a desirable trait without identification of all the genes that may control that trait. Thus, we chose to utilize the AFLP in its simplest form (i.e. without pre or post amplification restriction digests) for our study. Preliminary data using this approach had indicated that the DNA fingerprint of a sterile bull was uniquely different from bulls of average or high fertility (H.M. Zhang, personal communication). Those results led us to screen this larger population of bulls with known fertility outcomes based on DNA-based parentage verification of the offspring.

In addition, the amplified product that had been previously associated only with fertile bulls was cloned and sequenced. Nucleotide sequences obtained from two fertile bulls were compared, yielding a 50% homology over approximately 700 bp. The lack of homology between those sequences cannot be explained at this time. Even though the

reproducibility achieved by others using AFLP methodology was also high in this experiment, more sequence results are needed to verify the current findings. No nucleotide similarities were observed for the amplified product to the bovine TIMP-2 gene beyond the primer sequences, nor to other known genes. Over 200 bp of the obtained sequence had previously been identified in a bovine embryonic and extra-embryonic library (TIGR; Reference TC290499). The significance of that finding is not known at this time. Therefore, the data collectively presented herein represent novel bovine nucleotide sequence.

We utilized fertility data based upon parentage determination of the offspring to ascertain if this 700 bp gDNA product amplified by AFLP methodology was in fact a predictor of bull sterility. Although these data indicated there was a relationship to bull sterility with a three-fold greater percent probability when bulls where negative for the 700 bp marker, there was no difference in the mean number of calves sired by bulls whose gDNA resulted in an AFLP product compared to no AFLP product. Since approximately 22% of bulls sampled failed to produce the PCR-AFLP amplification product, this could represent a high number of breeding bulls annually if this population is representative of the United States beef industry.

Obviously other breeds beside Angus need to be screened for the frequency of the AFLP product, and fertility data need to be contrasted between animals whose DNA can or cannot be amplified by PCR to produce the 700 bp amplicon. An important question might be to determine what proportion of barren cows in a breeding population display or do not display the product analogues to the bulls sampled in this study. Furthermore, it

would be easy to identify animals with common ancestors in their pedigrees to evaluate whether the 700 bp AFLP product follows a pattern of Mendelian inheritance. Of course, due the heterogeneity of DNA sequences obtained from the two bulls in this study, more sequences will need to be compared in follow-up studies. Perhaps a smaller internal portion of the 700 bp fragment holds greater diagnostic utility and should serve as the amplicon for future selection experiments. It seems unlikely that this novel marker might explain the difference between sterile and fertile populations of bulls. Further investigations should also include segregating equal numbers of positive and negative bulls based on their AFLP 700 bp outcomes, and then evaluating the outcome on fertility in single-sire as well as multi-sire mating trials.

As previously mentioned, there are four proteins that can be used to predict bull fertility. The downfall of immuno-detection of seminal proteins is that fertility potential cannot be ascertained until bulls reach puberty. The development of genomic-based markers is needed to allow producers to select for increased fertility in both males and females at an early age. We have identified a novel, highly reproducible 700 bp genomic DNA marker that may prove useful to predict those individuals that may be more fertile. Future research should include screening females used for breeding since this marker is not gender-biased.

Figure 4.1. Representative genomic DNA PCR products from multiple bulls generated using forward 5'-GCGTTCTGCCTCCTGCTGCTG-3' and reverse 5'-CGCTTGATGGGGTTGCCGTAG-3' primers. Absence of the 700 bp band had been previously hypothesized to be an indicator of bull sterility. Lanes 1-4, 6 and 7 PCR products from separate Angus bulls; lane 5, 100 bp molecular ladder; lane 8, 175 bp PCR product generated using bovine cDNA template (PCR control).

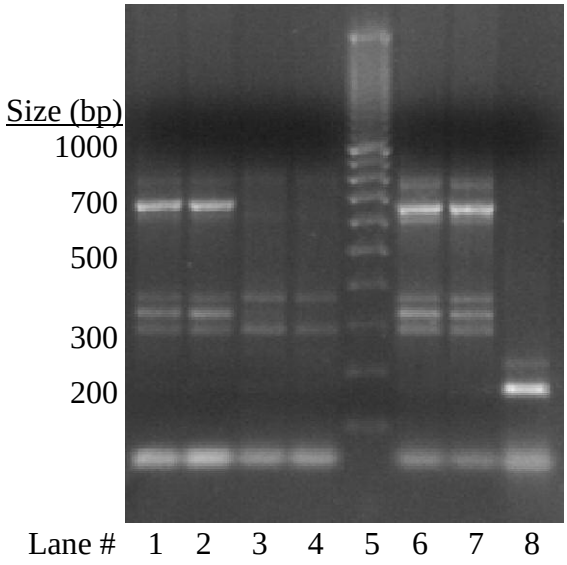


Figure 4.2. Distribution of bulls (N=55) categorized as 700 bp AFLP positive, negative or failed to amplify under defined conditions of the PCR reaction.

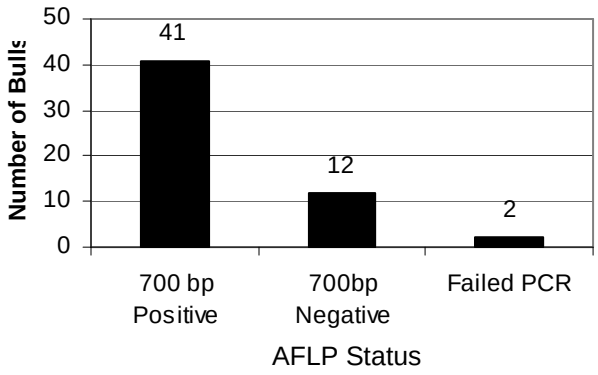


Figure 4.3. The mean (lsmean±SE) number of calves produced by bulls testing positive or negative for the 700 bp AFLP PCR product.

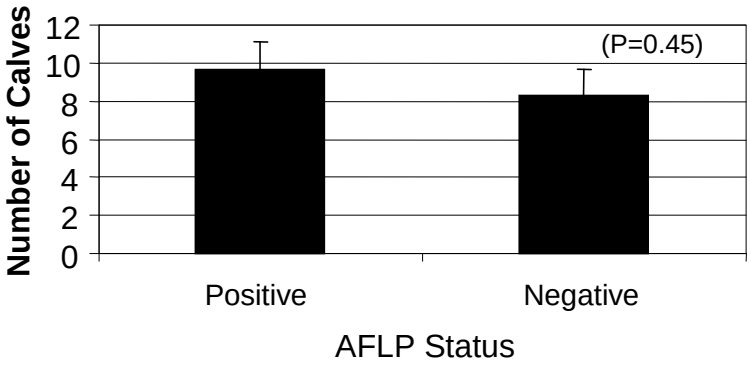
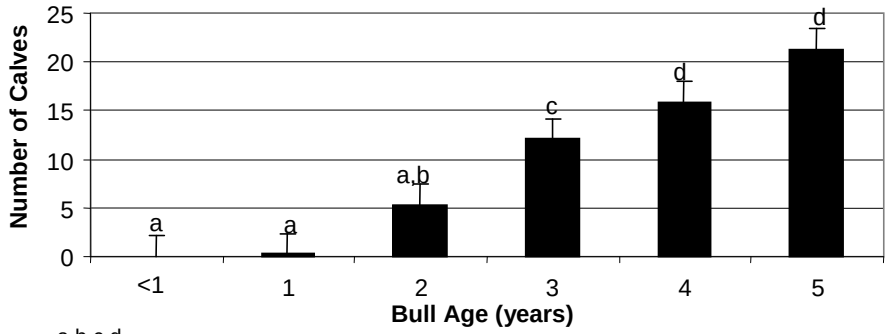
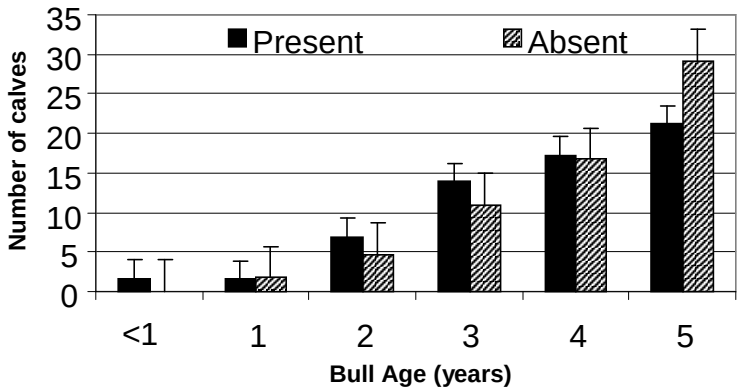


Figure 4.4. The mean (lsmean±SE) number of calves sired in relationship to bull age* (years).



a,b,c,d Means with different superscripts differ (P<.05).
*Age effects were significant (P<.0001).

Figure 4.5. The mean (lsmean±SE) number of calves produced in relationship to bull age (years) and presence or absence of the 700 bp AFLP PCR product.*



*No age by 700 bp band presence or absence interaction (P>0.7).

Table 4.1. Overall summary of reproductive performance[a] of Angus bulls screened for a 700 bp PCR product amplified from genomic DNA isolated from sperm.

	AFLP Product	
Overall Summary	Present	Absent
Number of Bulls	39	9
Total Breeding Opportunities[b]	117	27
Breeding Opportunities without a bull calf [c]	13	9
Percent (%) Breeding Opportunities without a bull calf	11.1%	33.3%
Total Bull Calves Born	1,270	283
Bull Calves Born/Breeding Opportunity	10.8	10.5

[a] Each bull was pastured at a ratio of 1 bull per 25 cows.
[b] Breeding opportunity was a 60 d breeding season for each bull replicated three consecutive breeding years.
[c] Number of bull calves sired/bull/year was based on DNA genotyping for parentage verification.

Figure 4.6. Multi-alignment of nucleotide sequences obtained from two separate Angus bulls identified as bull 070 and bull 055. A colon (:) identifies homologous nucleotides. After the vector sequence was removed, a 50.1 % identity was established between the sequences.

```
             10        20                  30         40
070   GCGTTCTGCCTCCTGCTGCTG------CAG---AGGGGCC-TCCGGGTGACACAAAGTG-
      ::::::::::::::::::::      ::    :   ::  :: :: :  ::   ::::
055   GCGTTCTGCCTCCTGCTGCTGTCTTTGCACTTTATTTGCAGTCTGGTTTTCAGGCAGTGG
             10        20        30        40        50        60

     50         60        70        80          90          100
070   ATCTG-CAGACGGTCCATTTCACCTCACCTACCCCA--GCCCATCCTTGGGGT----TCA
      : ::  :: ::  ::  :   :::: :   :  :::  :: : :  :  : ::    : :
055   AGCTTACACACAATCACTG--ACCTGAATAAGACCACTGCTCCTTTTCTGTGTGTGATGA
             70          80        90       100       110

                      110       120       130                140
070   ACTGA-----ATTAAGTGCTTTCTAACACCAGGC---------TTTAAAATAAAGAAAAC
      :::        : : :: ::  : :       ::           :::  : :   :  :
055   ACTCTGCACCAGTCAGCGCAGTATGGTCAGAGAAATGTTCTTGTTTCCAGTGTTGTCACA
     120       130       140       150       160       170
     150         160        170         180       190       200
070   GCTTAG--CTGTCTG-GTTATTCAAA--TAGTTTGTGAATGGGACAACCTGGGACAATCG
      ::::    ::::::  :::: : :::  :: : : :::::: : : ::  ::  : : : :
055   GCTTGTGTCTGTCTAAGTTAGTAAAAAATATTCTTTGAATTGAATAAAATGAAA-ACTGG
     180       190       200       210       220       230

          210       220       230       240       250       260
070   CTTCATCTTAGGCCAGGCCTGTGCTGCTGACTTCTCATCATGGGAGCAGCGCTCCAAGTC
         : ::::    : ::  :::: :::   :::   :  : ::: :: : :    :  ::
055   AGGCCTCTTGTTTCTGG--TGTGGTGCCCCCTTGG-AGGAGGGGGGCTGGGGGTGAGCTC
      240       250          260       270         280       290

           270         280       290       300          310
070   AAG-TCCTGTCTCAG--AGCATCTTCTGAACAGGCCACCATCCCT---GCCATGACCCCA
      :   : :: :  : :  :: : ::   :  ::::  : :::::     :: :: :
055   ACCCTACTCTGGCGGCCAGAAGCTG--GGGCAGGGGAGCATCCTGAGGGCTATAAGTGGG
         300       310          320       330       340       350

      320       330       340       350         360       370
070   CCCACTTATAGCCCTCAGGATGCTCCCCTGCCCC--AGCTTCTGGCCGCCAGAGTAGGGT
           : :: ::     ::::: :  ::::  :   :: : ::  : :  : :: :   :
055   TGGGGTCATGGCAG---GGATGGTGGCCTGTTCAGAAGATGCT--CTGAGACAGGACT-T
           360          370       380       390         400

        380       390         400       410         420       430
070   GAGCTCACCCCCAGCCCCCCTC-CTCCAAGGGGGCACCACA--CCAGAAACAAGAGGCCT
      ::  :    : : :: ::: :  :   :::   ::: ::::  :: :    :: : :
055   GACTTGGAGCGCTGCTCCCATGACGAGAAGTCAGCAGCACAGGCCTGGCCTAAAATGAAG
       410       420       430       440       450       460
```

```
          440        450       460       470       480       490
070   CCAGTTTTC-ATTTTATTCAATTCAAAGAATATTTTTTATTAACTTAGACAGACACAAGA
      : : : : : : ::: : : ::::: : : :::::   : ::::  ::::::
055   CGATTGTCCCAGTTTGTCCCATTCACA-ACTATTTG--AATAACC-AGACAG--------
                470       480       490        500          510

            500       510       520       530       540       550
070   TGTGACAACACTGGAAACAAGAACATTTCTCTGACCATACTGCGCTGACTGGTGCAGAGT
                ::      :::  : ::::: : :   ::  ::     :::: : :::
055   ----------CT------AAG--CGTTTCT-TTATTTTAAAGC-----CTGGGGTAGAAA
                           520        530            540       550

            560       570       580       590       600       610
070   TCATCACACACAGAAAAGGAGCAGTGGTCTTATTCAGGTCAGTGATTGTGTGTAAGCTCC
       :: :  :  :::   :    ::  : :    :   ::   ::::  :::  :  :  :
055   GCA-CTAATTCAGTTGAACCCCAAGGATGGGCTGGGGGAAGGTGAG-GTGAATGGGACCG
              560       570       580       590        600

            620       630       640       650       660       670
070   ACTGCCTGAAAAACAGACTGCAAATAAAGTGCAAAGACAGCAGCAGAGGCAGAACGCAGG
        :::    :  ::    ::  :    ::  :     ::::::  :::::::: ::::::
055   GTTGCG---ATCACTTTGTGGCACCGGAGGCCCTCTGCAGCAGAGGAGGCAGA-CGCAGG
     610          620       630       640       650        660

            680       690
070   GCAATTCGAAGCTTGAAGGTAACCTA
      :::::: :::::: :: :  :: :::
055   GCAATTTGAAGCTGGAGG--AAGCTA
```

CHAPTER FIVE

I. An Equine Homologue to the Gene For Bovine Fertility-Associated Antigen (FAA)[a]

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A. Abstract

Efforts are underway to identify biomarkers in semen that may serve to predict fertility of males. One protein, designated as fertility-associated antigen (FAA) has been characterized from bovine semen. It is a 31 kDa non-glycosylated peptide produced in all of the accessory sex glands. Monoclonal and polyclonal antibodies have been developed to screen for FAA, and bulls with detectable FAA using an antibody-based detection system are more fertile than herdmates producing semen without detectable FAA. Using immunofluorescence, FAA has been detected on surfaces of sperm from bulls, rams and humans, but not on sperm from stallions. Since FAA is a heparin-binding protein, and stallion sperm display variable in vitro responses to heparin-induced capacitation, the purpose of this research was to determine whether an equine genetic homologue to bovine FAA could be amplified or detected in stallion accessory gland tissue. Reported herein, is a unique equine nucleotide sequence, which is approximately 88.5% identical to the bovine consensus sequence encoding for the FAA gene. Translated, the amino acid sequence maintains 86% identity to bovine FAA (bFAA). These results suggest that subtle differences between the equine and bFAA gene account for the inability to detect the protein in stallion semen using antibody-based diagnostics. Moreover, this research describes a genetic variant of a well-characterized, high-affinity bovine heparin-binding protein (HBP).

B. Introduction

Heparin, a sulfated glycosaminoglycan (GAG), which is most well-known for its anti-coagulative properties in blood, can effectively promote capacitation of bovine (Parrish et al., 1988 and 1989), ram (Slavik et al., 1990; Slavik and Fulka, 1991), human (Roy et al., 1985) and stallion sperm (Blue et al., 1989; Farlin et al., 1993; Varner et al., 1993; Christensen et al., 1996 and Merkies and Buhr, 1998) *in vitro* and *in vivo* (Fleet et al., 1995). However, the response of stallion sperm to heparin is variable and dependent on the concentration of heparin (range 10-100 ug/ml), incubation intervals and inducers or stimulators, which have primarily been non-physiological (i.e. calcium ionophore, A23187), for the final induction of the acrosome reaction (reviewed by Graham, 1996). Similar to ruminants (Lee and Ax, 1984 and Lee et al., 1986), stallion sperm appear to be exposed to heparin-like material (or GAGs) in the reproductive tract of mares, as those constituents have been isolated and concentrations appeared to be highest during estrus (Varner et al., 1991).

Numerous seminal proteins have been identified in mammalian semen; however, their role or biological function in stallion semen remains to be elucidated. Calvete et al. (1994; 1995) biochemically identified a series of horse seminal plasma proteins (HSP; 1-8), of which, HSP-1 and HSP-2 were major heparin-binding proteins (HBP) while HSP-7, also an HBP, was similar to spermadhesin AWN-1-like protein (Sanz et al., 1992), a zona pellucida binding protein in boar sperm. Others have attempted to identify seminal protein patterns using two-dimensional gel electrophoresis to correlate those protein patterns to stallion fertility by regression analysis (Brandon et al., 1999). Antibodies

developed against a 55 kDa, bovine fertility-associated protein (Killian et al., 1993) later determined to be osteopontin (OPN; Cancel et al., 1997) demonstrated antigenic properties against a 72-kDa stallion protein (known as SP-1) and was further found to have a positive relationship (r^2=.706) to stallion fertility (Brandon et al., 1999). However, the bovine fertility-associated protein, OPN, has not been shown to bind heparin unlike the bovine HBPs, fertility-associated antigen (FAA; McCauley et al., 1999) and type-2 tissue inhibitor of metalloproteinase (TIMP-2; Calvete et al., 1996 and McCauley et al., 2001). Both FAA and TIMP-2 have been purified using heparin-affinity chromatography, and FAA has been used as a predictor of bull fertility (Bellin et al., 1994, 1996, 1998; and Sprott et al., 2000). Those proteins are produced by the accessory sex glands (Miller et al., 1990). Also, immunolocalization preformed with the monoclonal antibody M1 (Bellin et al., 1996) detected those proteins on ejaculated sperm, but not on epididymal sperm (McCauley et al., 1996), which indicated that they bind to sperm upon ejaculation as sperm transverse the male reproductive tract or come in contact with seminal constituents. Heparin is believed to interact with specific HBPs and potentiate heparin-induced capacitation. Since FAA expresses the highest affinity among the five identified families to bind heparin (Miller et al., 1990), and binding affinity is indicative of bull fertility potential (Marks and Ax, 1985), the absence of FAA in stallion semen may partially explain why stallion sperm cultured with heparin *in vitro* respond more variably to a given dose of heparin.

To date, FAA has not been detected in stallion semen or on sperm (Dawson et al., 2003) using a monoclonal antibody (M1) or with anti-recombinant FAA (anti-rFAA;

McCauley et al., 2004) rabbit polyclonal antibodies. The absence or inability to detect FAA in stallion semen led to the objectives of this experiment, which were 1.) To amplify the equine FAA gene from accessory sex gland cDNA, 2.) To compare nucleotide and amino acid sequences to bFAA and 3.) Verify the presence or absence of FAA using protein extracts from equine accessory sex gland tissue.

C. Materials and Methods

All chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated.

1. Tissue Harvest

A highly fertile, 21 year old stallion maintained at the College of Veterinary Medicine, Texas A&M University (College Station, TX 77843) served as the tissue donor for these following experiments. The stallion was scheduled for euthanasia due to physical complications. Upon euthanasia, tissue samples were removed via aseptic technique from the following regions of the reproductive tract: ampulla, bulbourethral, prostate and seminal vesicle glands, epididymis, and testis. Liver tissue was also harvested for comparison purposes. Tissue samples were removed from each gland and placed into 2-ml cryovials and snap-frozen on dry ice. All samples were then shipped overnight to the University of Arizona (Tucson, AZ 85721) on dry ice and then stored at -80^{o} C until RNA or protein isolation.

2. RNA Isolation

Isolation of RNA was performed using TRIzol® reagent (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Briefly, frozen tissue samples (300 mg each) from

liver, bulbourethral, prostate and seminal vesicle glands were placed directly into 5 ml TRIzol® reagent. Individual tissue samples were then homogenized on ice using a variable speed Tissue Tearor™ (Biospec Products, Inc., Racine, WI). Homogenized samples were incubated for 5 min at room temperature (RT) for phase separation to occur, and then, 1 ml chloroform was added. Samples were then briefly vortexed and incubated at RT for an additional 5 min. All samples were then centrifuged at 12,000xg for 15 min at 4^{o} C to partition the aqueous phase. The aqueous or RNA phase was aspirated from each sample and transferred to a separate tube. Isopropyl alcohol (2.5 ml) was then added to the aqueous phase to precipitate RNA. Following 10 min incubation at RT, samples were again centrifuged at 12,000xg for 10 min at 4^{o} C. The supernatant was discarded and the RNA pellet washed once with 5 ml of 75% ethyl alcohol (EtOH) and centrifuged at 7,500xg for 5 min at 4^{o} C. The supernatant was again removed, the RNA pellet was allowed to briefly air-dry, and was then resuspended with sterile DEPC water (EMD Chemicals, Gibbstown, NJ). RNA was quantified and purity ($A_{260/280}$) assessed using a spectrophotometer (GeneQuant II; Pharmacia Biotech, Cambridge, England) and intact 28S and 18S bands were visualized by agarose gel electrophoresis before reverse transcription and first strand cDNA synthesis.

3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Prior to PCR, 1 ug of isolated total RNA from each separate gland was treated with amplification grade deoxyribonuclease I (DNase I; Invitrogen) to eliminate potential DNA contamination in follow-on procedures. This was performed by incubating RNA for 15 min at RT with 1 U of DNase I (activity >10,000 U/mg) in a 10 ul reaction

containing 20 mM Tris-HCl (pH=8.4), 2 mM magnesium chloride ($MgCl_2$) and 50 mM potassium chloride (KCl). DNase I activity was quenched after 15 min by addition of 1 ul 25 mM ethylenedinitrilo-tetraacetic acid (EDTA) and heating for 10 min at 65^o C in an Eppendorf Thermomixer 5436 (Brinkmann Instruments, Westbury, NY).

Reverse transcription PCR was then performed to amplify first strand cDNA from the bulbourethral, prostate, seminal vesicle and liver RNA samples utilizing the Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) in conjunction with oligo $(dT)_{20}$ primers. Five-hundred ng of RNA were used in each reaction following manufacturer's suggested protocol. All RT-PCR incubations were carried out in a Peltier Thermal Cycler 200 (PTC-200; MJ Research, Inc, Watertown, MA). All products were stored at -20^o C.

4. Primer Design

Bovine primers originally designed based on bovine FAA amino acid sequence (McCauley et al., 1999) were used unsuccessfully in an attempt to amplify FAA from stallion accessory gland tissue. Therefore, novel primers were custom synthesized (Invitrogen Custom Primers, Carlsbad, CA) based on the 900 bp consensus sequence of bovine FAA, a public 99% identical EST (tc229851) sequence through The Institute for Genomic Research (TIGR; http://www.tigr.org/) and a highly similar (~87%) *Homo sapien* deoxyribonuclease I-like 3 (hDNase1L-3; Genbank accession NM_004944). Those primer sequences are listed below.

Forward 5'-CATGAGGATCTGCTCCTTCAA-3'

Reverse 5'-AACTGGAAAGTGGTCGCTGA-3'

5. PCR Amplification of FAA

The cDNA generated from the accessory sex glands and liver were used separately in 25 ul PCR reactions consisting of 10mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton® X-100, 1.5 mM $MgCl_2$, 200 µM each of dATP, dTTP, dCTP and dGTP, 0.5 µM of each gene specific primer and 1.25 units of *Taq* DNA polymerase per reaction (all obtained from Promega Corporation, Madison, WI) in a 200 µl thin wall PCR tube (Bio-Rad, Hercules, CA). The thermocycler (PTC-200) conditions were 94°C, 58°C and 72°C for 1 min each for 40 cycles with a 15 min final extension at 72^o C followed by maintenance at 4^o C. Samples were removed and PCR products prepared for electrophoresis. A 10-ul aliquot from each reaction was removed and placed into a separate .5 ml microcentrifuge tube to which 6X loading buffer (components of Load dye) was added. All samples were loaded in to a 2% (wt/vol) agarose gel containing 90 mM Tris, 90 mM boric acid, 2 mM EDTA (TBE buffer, pH=8.3) with ethidium bromide (EtBr; 5ug/ml). All gels were electrophoresed in a horizontal gel apparatus (Bio-Rad) at 75 V for 20 min and followed by 100 V until complete. A 100 bp PCR DNA ladder (EZ Load 100 bp Molecular Ruler, Bio-Rad) served as reference standard. Gel images were captured using an ultraviolet light box and CCD camera linked to Alpha Imager™ software (Alpha Innotech Corporation, San Leandro, CA).

6. Cloning and Sequencing of PCR Products

Fresh PCR products from the seminal vesicle and liver were directly cloned into the pCR®4-TOPO® vector using the TOPO® TA cloning kit (Invitrogen) following manufacturer's recommendations. Cloning reactions were allowed to proceed for 15

min. Transformations of cloned products were performed using TOP10 One Shot® chemically competent E. coli (Invitrogen). Transformation reactions were incubated on ice for 30 min and then heat-shocked for 30 seconds at 42° C. Room temperature SOC medium (250 ul; Invitrogen) was then added to each transformation and placed in to an Environ Lab-line shaker (Barnstead International, Dubuque, IA) at 37° C and 200 rpm. Cultures were maintained for 30 min in the incubator and then 50 and 100 ul aliquots were plated on separate pre-warmed selective Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 1.5% agarose, pH=7.0) plates supplemented with 50 ug/ml of kanamycin antibiotic. Two volumes were plated to ensure an adequate number of well-spaced colonies for selection purposes. Plates were incubated at 37° C overnight.

Multiple colonies from each transformation were then selected and cultured in LB media (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH=7.0) with kanamycin (50 ug/ml) for an additional 12 h using a shaking (225 rpm) 37° C incubator. Bacterial cells were then harvested by centrifugation at 1,000xg for 10 min at RT. Cells were lysed and plasmids purified using the Qiagen Spin Miniprep columns (Qiagen, Valencia, CA) according to manufacturer's instructions. PCR was performed on purified plasmids to validate the presence of an insert corresponding to the bp size of the cloned product using standard vector supplied amplification primers.

Insert Re-PCR was performed by using Promega Master Mix (Promega) per manufacturer's directions with 10 ng of plasmid DNA template per reaction. The cycling conditions were 94°C, 55°C and 72°C for one min each repeated 35 times on a PTC-200 thermocycler. PCR products were visualized as previously described for agarose gel

electrophoresis. Plasmids, which were successfully screened and maintained a discrete single PCR band of the correct size, were then sent to the University of Arizona DNA sequencing facility. All samples were sequenced using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence analyses and comparisons were performed using The Biology WorkBench version 3.2 software available at http://workbench.sdsc.edu/.

7. Protein Extraction, Gel Electrophoresis and Western blotting

Tissue extracts used for anti-recombinant FAA (rFAA) polyclonal immunoblotting assay were performed by homogenizing tissue as previously described herein using buffers adapted from Oughtred et al. (2002). Five-hundred mg of each tissue from the bulbourethral, prostate, seminal vesicles and liver were homogenized on ice in 5 ml of extraction buffer consisting of 25 mM Tris (pH=7.5), 2% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT), and protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin A (10ug/ml) and leupeptin (10ug/ml). All samples were then centrifuged at 10,000xg for 30 min at 4^{o} C. Supernatants were removed by aspiration and protein concentration determined by the DC Protein Assay kit (Bio-Rad) with bovine serum albumin (BSA) as the standard.

Tissue extracted proteins were then diluted into sample loading buffer (10% glycerol, 2% SDS, 0.001% bromophenol blue in 65 mM Tris) and boiled for 5 min (as described by Bellin et al., 1996). Fifty ug of total protein were loaded per lane on a 12 % SDS-polyacrylamide gel (SDS-PAGE). Duplicate gels were performed for Coomassie brilliant blue G protein staining and immunoblotting.

Gels used for immunoblotting were electrophoretically transferred onto 0.2 μm nitrocellulose membrane (Trans-Blot® Transfer Medium; Bio-Rad) using a graphite electroblotter (Millipore Corporation, Bedford, MA). Membranes were stained with 0.1% Ponceau S containing 5% acetic acid and then blocked with 5% BSA (wt/vol) in phosphate-buffered saline (PBS) overnight at 4^{o} C. Membranes were washed three times in PBS-1% BSA and incubated with anti-rFAA rabbit serum diluted 1:1000 in PBS-3% BSA for 4 h at RT on a rocker. Again, blots were washed with three changes of PBS-1% BSA and anti-rabbit goat alkaline phosphatase (AP)-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1.5 h at RT. Following secondary antibody incubation, membranes were rinsed in 150 mM NaCl, 50 mM Tris-base (pH=7.5) for 10-15 min. Colorimetric detection was then performed using nitro blue tetrazolium (NBT) and 5-bromo-4chloro-3-indolyl phosphate (BCIP) in 70% and 100% dimethyl formaldehyde (final concentration 50mg/ml) added at 66 ul and 34 ul, respectively, to 10 ml Western blot reaction buffer (150mM NaCl, 100mM Tris-base and 5mM $MgCl_2$; pH=9.4). Development was allowed to proceed for 30 min and stopped by rinsing with dd H_20. Western blots were air-dried and digitally photographed as described previously.

D. Results

1. Amplification and Sequence Analysis of Equine FAA (eFAA)

PCR with primers originally designed from nucleotide sequence derived from peptide digestion assays of bovine FAA (McCauley et al., 1999) were unsuccessful in amplifying those particular regions of eFAA (data not shown). Therefore, new primers

were designed as described above and were employed to amplify eFAA from cDNA derived from the stallion accessory sex glands. The forward, 5'-CATGAGGATCTGCTCCTTCAA-3' and reverse, 5'-AACTGGAAAGTGGTCGCTGA-3, primers were designed to amplify a 770 bp PCR product from cDNA. Figure 1 shows PCR amplification of a discrete single 800 bp band in all lanes 2-5, from the liver, bulbourethral, prostate and seminal vesicular glands, respectively.

PCR products (Figure 1) from the seminal vesicles and liver were cloned and nucleotide sequences obtained. Figure 2 illustrates the homology of a 771 bp product amplified from the seminal vesicles and liver in a multi-sequence alignment. Only two single bp mismatches were identified at positions 707 (clone eSV-2) and 735 (clone eLiver-1). Nucleotide consensus sequence from Figure 2 was 89% identical to *Homo sapien* deoxyribonuclease I-like 3 (hDNase IL3; accession NM_004944.1). This finding is relevant for the nucleotide-nucleotide comparison (Figure 3) of bovine and equine FAA since the consensus 900 bp nucleotide sequence of bFAA shares an 88.8% identity to hDNase IL3. The nucleotide alignment of equine FAA to bFAA (Figure 3) possesses a 749 nucleotide overlap, of which, there is an 88.5% identical consensus that begins at bp 153 and spans through the 3' end of the bFAA sequence.

Translated amino acid sequences of eFAA and bFAA are presented in Figure 4. A high homology exists with a 243-residue overlap, of which, 210 amino acid residues are identical (86%) and 226 of the 243 (93%) residues are considered conserved or positive substitutions. Taking this into account, eFAA and bFAA amino acid sequences

only differed by 7%. However, the majority of those discrepancies were found to be toward the carboxyl terminus of the peptide, where homology dramatically declined.

2. Western blotting of Tissue Extracts

Tissue from the bulbourethral, prostate, seminal vesicles and liver were used in an immunoblotting experiment to verify the presence or absence of FAA in equine tissue. Bovine seminal vesicle tissue served as the positive control for comparison purposes. The anti-rFAA Ab successfully detected FAA in bovine tissue (Figure 5; lane 1). However, no distinct band of the same molecular weight was observed in equine bulbourethral, prostate, seminal vesicle or liver tissue, respectively (lanes 3-6). Non-specific background was elevated in these results, due to secondary antibody cross-reactivity with tissue samples under lengthy exposures. However, some smaller (less than 20 kDa) bands exhibited intense cross-reactivity above background, suggestive of some partial hydrolysis of a mature FAA peptide or epitopic cross-reactivity with other peptides.

E. Discussion

The lack of cross-reactivity of stallion semen/sperm extracts with the anti-FAA antibodies, both polyclonal and monoclonal, suggests that subtle differences may exist between the FAA gene in equine compared to bovine, accounting for the inability to detect the protein. This, coupled with the additional variable and controversial results with supplementing heparin in culture to promote capacitation of stallion sperm is intriguing. If a specific, high-affinity heparin binding protein (FAA) is absent or

produced as a different isoform, this provides a potential partial explanation for variations between bull and stallion sperm incubated with heparin to potentiate capacitation *in vitro*.

The internal bFAA peptide digestion assays (McCauley et al., 1999) yielded peptides which ranged in identity to hDNase1L-3 from 73 to 92%. Since that initial discovery, a 900 bp nucleotide consensus sequence has been identified for bFAA (Ax et al., 2003) with approximately 88% nucleotide identity to hDNase1L-3 (Accession NM_004944) and 99% (898/900 bp) identity to a bovine EST sequence (tc229851). Bovine specific primers, originally constructed from peptide digestions were unsuccessful in amplifying products from stallion accessory gland cDNA (or liver, data not shown). Using the consensus bFAA sequence and two highly homologous sequences, a bovine EST and hDNase1L-3 originally discovered in 1997 (Rodriguez et al.; and Zeng et al.) new primers were constructed. Those primers successfully amplified a single, specific 800 bp product from the three separate accessory sex glands and the liver. Those products were cloned and clones from the seminal vesicles and liver were sequenced to verify identity of the amplified product. Nucleotide sequences obtained from those clones were 88.5% identical to bFAA (Figure 3), which also predicts a translated amino acid sequence (Figure 4) that is 86% identical. The majority of non-conserved amino acid residues existed near the carboxyl terminus of the translated peptide sequence. The relevance of this finding is still uncertain, however, it is reasonable to hypothesize that mutations near this region of the bovine gene have resulted in the inability to detect FAA in semen from those males (unpublished data) using antibody-based detection methods. Antibody-epitope mapping to determine which

antigenic sites of the FAA molecule are required for recognition by the antibodies will have to be deduced before this observation can be concluded.

FAA in stallion semen may undergo hydrolysis as it is produced by the accessory sex glands, thereby preventing its detection after ejaculation. To examine this hypothesis, immunoblots were performed using protein extracts from stallion accessory sex glands and the seminal vesicular gland from a bull (positive control) with the anti-rFAA polyclonal antibody. Non-specific binding was higher than desired with those tissue extracts, however, cross-reactivity with FAA in bovine tissue (Figure 5) was apparent, while no discrete single band of the same molecular weight was present in stallion tissue. This finding, in conjunction with differences in the amino acid predictions of eFAA, suggests that an aberrant form of FAA is produced by the stallion and immuno-detection methods developed for bovine are insufficient to recognize FAA from the stallion.

Fertility-associated antigen is similar to a DNase I homologue, human DNase1L-3 (Rodriguez et al., 1997; Zeng et al., 1997) and further characterized by Yakovlev et al., (1999 and 2000). DNase I activity has been confirmed in mammalian semen (Yasuda et al., 1993), which is proposed to modulate germ cell numbers by apoptosis (Stephan et al., 1996) by inducing DNA degradation (Peitsch et al., 1993). However, FAA (bovine and equine) is only approximately 50% homologous to DNase I while it shares almost 90% homology with hDNase 1L-3. DNase 1L-3 activity, to our knowledge, has not been reported in semen, but nevertheless it does possess some key biological properties. Those include both a calcium-binding and DNA-binding domain along with sites for

signal peptide cleavage (Rodriguez et al., 1997). The sequestering of intracellular calcium by sperm is one, clear prerequisite to capacitation and the subsequent acrosome reaction (for reviews see Breitbart, 2002a; 2002b). Therefore, a unique protein (i.e., FAA) or novel isoform of DNase 1L-3 may participate in this physiological event by assisting in the elevation of intracellular calcium levels leading to the observed acrosome reaction.

In conclusion, elucidating the underlying molecules which may interact with heparin in stallion semen may provide a valuable means of altering seminal (or media) constituents to enhance capacitation and the acrosome reaction, improving *in vitro* or *in vivo* fertilizing capabilities.

Figure 5.1. Amplified PCR products from oligo $(dT)_{20}$ stallion cDNA template using forward 5'-CATGAGGATCTGCTCCTTCAA-3' and reverse 5'-AACTGGAAAGTGGTCGCTGA-3' primers generated from comparing bovine and human homologous sequences. PCR conditions were 94°C, 58°C and 72°C for 1 min each with 40 cycles. Lane 1, DNA ladder; lanes 2-5 PCR products generated from stallion liver, bulbourethral, prostate and seminal vesicular glands, respectively. PCR products were cloned and sequenced as described in Materials and Methods.

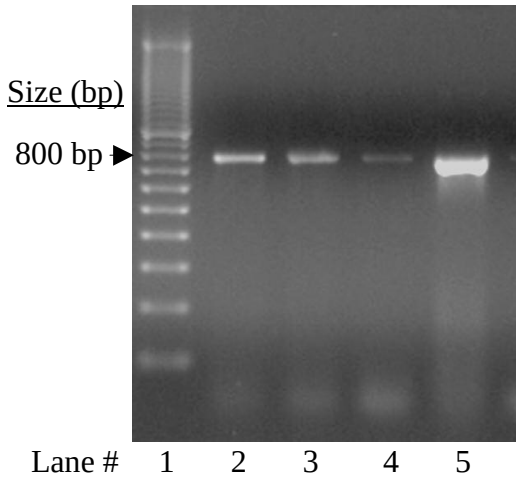

Figure 5.2. Multi-sequence alignment of cloned PCR products from equine seminal vesicles (eSV) and liver. Two bp mismatches were identified (see arrowheads) at positions 707 (clone eSV-2) and 735 (clone eLiver-1).

```
          1                                                          50
   eSV-1  CATGAGGATC TGCTCCTTCA CGTGAGGTCC TTTGGGGAAT CCAAGAAGGA
   eSV-2  CATGAGGATC TGCTCCTTCA CGTGAGGTCC TTTGGGGAAT CCAAGAAGGA
ELiver-1  CATGAGGATC TGCTCCTTCA CGTGAGGTCC TTTGGGGAAT CCAAGAAGGA

          51                                                        100
   eSV-1  AAATCGGAAC ACCATGGACG TCATTGTGAA GGTCATCAAA CGCTGTGACA
   eSV-2  AAATCGGAAC ACCATGGACG TCATTGTGAA GGTCATCAAA CGCTGTGACA
ELiver-1  AAATCGGAAC ACCATGGACG TCATTGTGAA GGTCATCAAA CGCTGTGACA

          101                                                       150
   eSV-1  TCATACTCCT GATGGAAATC AAGGACAGTA ACAACATGAT CTGTCCCACG
   eSV-2  TCATACTCCT GATGGAAATC AAGGACAGTA ACAACATGAT CTGTCCCACG
ELiver-1  TCATACTCCT GATGGAAATC AAGGACAGTA ACAACATGAT CTGTCCCACG

          151                                                       200
   eSV-1  CTGATGGAGA AGCTGAACGG AAATTCAAGA AGAGGCATAA CATACAACTA
   eSV-2  CTGATGGAGA AGCTGAACGG AAATTCAAGA AGAGGCATAA CATACAACTA
ELiver-1  CTGATGGAGA AGCTGAACGG AAATTCAAGA AGAGGCATAA CATACAACTA

          201                                                       250
   eSV-1  TGTGATTAGC TCTCGGCTCG GAAGAAACAC ATATAAAGAA CAATATGCCT
   eSV-2  TGTGATTAGC TCTCGGCTCG GAAGAAACAC ATATAAAGAA CAATATGCCT
ELiver-1  TGTGATTAGC TCTCGGCTCG GAAGAAACAC ATATAAAGAA CAATATGCCT

          251                                                       300
   eSV-1  TTCTCTACAA GGAAAAGTTA GTGTCTGTGA AGAAAAGATA CCTCTACCAT
   eSV-2  TTCTCTACAA GGAAAAGTTA GTGTCTGTGA AGAAAAGATA CCTCTACCAT
ELiver-1  TTCTCTACAA GGAAAAGTTA GTGTCTGTGA AGAAAAGATA CCTCTACCAT

          301                                                       350
   eSV-1  GACTATCAGG CTGGAGACGC AGATGTATTT TCCAGGGAGC CCTTTGTGAT
   eSV-2  GACTATCAGG CTGGAGACGC AGATGTATTT TCCAGGGAGC CCTTTGTGAT
ELiver-1  GACTATCAGG CTGGAGACGC AGATGTATTT TCCAGGGAGC CCTTTGTGAT

          351                                                       400
   eSV-1  CTGGTTCCAG TCACCCTACA CCGCTGTCAA GGACTTTGTG ATTGTCCCCC
   eSV-2  CTGGTTCCAG TCACCCTACA CCGCTGTCAA GGACTTTGTG ATTGTCCCCC
ELiver-1  CTGGTTCCAG TCACCCTACA CCGCTGTCAA GGACTTTGTG ATTGTCCCCC

          401                                                       450
   eSV-1  TGCACACCAC CCCTGATACA TCCGTTAAAG AGATTGATGA GCTGGCTGAT
   eSV-2  TGCACACCAC CCCTGATACA TCCGTTAAAG AGATTGATGA GCTGGCTGAT
ELiver-1  TGCACACCAC CCCTGATACA TCCGTTAAAG AGATTGATGA GCTGGCTGAT
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```
          451                                                        500
   eSV-1  GTCTACGTGG ACGTGAAACG CCGTTGGAAG GCAGAGAATT TCATTTTCAT
   eSV-2  GTCTACGTGG ACGTGAAACG CCGTTGGAAG GCAGAGAATT TCATTTTCAT
ELiver-1  GTCTACGTGG ACGTGAAACG CCGTTGGAAG GCAGAGAATT TCATTTTCAT

          501                                                        550
   eSV-1  GGGTGACTTC AACGCCGGCT GCAGCTACGT CCCCAAGAAG GCCTGGAAGA
   eSV-2  GGGTGACTTC AACGCCGGCT GCAGCTACGT CCCCAAGAAG GCCTGGAAGA
ELiver-1  GGGTGACTTC AACGCCGGCT GCAGCTACGT CCCCAAGAAG GCCTGGAAGA

          551                                                        600
   eSV-1  ACATCCGCCT GAGAACCGAC CTTGGGTTTG TTTGGCTGAT CGGGGATGAA
   eSV-2  ACATCCGCCT GAGAACCGAC CTTGGGTTTG TTTGGCTGAT CGGGGATGAA
ELiver-1  ACATCCGCCT GAGAACCGAC CTTGGGTTTG TTTGGCTGAT CGGGGATGAA

          601                                                        650
   eSV-1  GAGGACACCA CGGTGAAGAG CAGCACCAAG TGTGCATATG ACAGGATTGT
   eSV-2  GAGGACACCA CGGTGAAGAG CAGCACCAAG TGTGCATATG ACAGGATTGT
ELiver-1  GAGGACACCA CGGTGAAGAG CAGCACCAAG TGTGCATATG ACAGGATTGT

          651                                                        700
   eSV-1  GCTTAGAGGA CGAGAGATTG TCAGCTCTGT TGTTCCCAAA TCAAACAGCG
   eSV-2  GCTTAGAGGA CGAGAGATTG TCAGCTCTGT TGTTCCCAAA TCAAACAGCG
ELiver-1  GCTTAGAGGA CGAGAGATTG TCAGCTCTGT TGTTCCCAAA TCAAACAGCG

          701   ▼                                    ▼             750
   eSV-1  TCTTCAACTT CCAGAAATCT TATGCTTTGA CTGAAGAGGA GGCCCTGGGT
   eSV-2  TCTTCA.CTT CCAGAAATCT TATGCTTTGA CTGAAGAGGA GGCCCTGGGT
ELiver-1  TCTTCAACTT CCAGAAATCT TATGCTTTGA CTGA.GAGGA GGCCCTGGGT

          751                 771
   eSV-1  GTCAGCGACC ACTTTCCAGT T
   eSV-2  GTCAGCGACC ACTTTCCAGT T
ELiver-1  GTCAGCGACC ACTTTCCAGT T
```

Figure 5.3. Nucleotide sequence alignment of bovine fertility-associated antigen (bFAA) and equine FAA (eFAA). Identical bp matches are indicated by a colon (**:**). A 749 nucleotide overlap existed between bFAA and eFAA sequences corresponding to an overall identity of 88.5%.

```
            160       170       180       190       200       210
bFAA   CCTCAAGATCTGCTCCTTCAATGTGAGGTCCTTTGGGGAATCCAAGAAGGCAAACTGTAA
       : : : :::::::::::: : ::::::::::::::::::::::::::::: :::  : ::
eFAA   CATGAGGATCTGCTCCTTC-ACGTGAGGTCCTTTGGGGAATCCAAGAAGGAAAATCGGAA
               10         20        30        40        50

            220       230       240       250       260       270
bFAA   TGCCATGGATGTCATTGTGAAGGTCATCAAACGCTGTGATATCATACTCCTGATGGAAAT
         ::::::: :::::::::::::::::::::::::::: ::::::::::::::::::::
eFAA   CACCATGGACGTCATTGTGAAGGTCATCAAACGCTGTGACATCATACTCCTGATGGAAAT
      60         70        80        90       100       110

            280       290       300       310       320       330
bFAA   CAAGGACAGCAGCAACAGGATCTGCCCCACACTGATGGAGAAGCTAAACGGAAATTCAAG
       ::::::::: : ::::: ::::::: ::::: :::::::::::::: ::::::::::::::
eFAA   CAAGGACAGTAACAACATGATCTGTCCCACGCTGATGGAGAAGCTGAACGGAAATTCAAG
     120       130       140       150       160       170

            340       350       360       370       380       390
bFAA   AAAAGGCATAACATACAACTATGTGATTAGCTCTCGCCTTGGAAGAAACACATATAAAGA
       :: ::::::::::::::::::::::::::::::::: :: ::::::::::::::::::::
eFAA   AAGAGGCATAACATACAACTATGTGATTAGCTCTCGGCTCGGAAGAAACACATATAAAGA
     180       190       200       210       220       230

            400       410       420       430       440       450
bFAA   ACAGTATGCCTTTCTCTATAAAGAAAAGCTAGTGTCTGTAAAACAAAGCTACCTCTACCA
       ::: :::::::::::::: :: :::::: :::::::::: ::  :::: ::::::::::
eFAA   ACAATATGCCTTTCTCTACAAGGAAAAGTTAGTGTCTGTGAAGAAAAGATACCTCTACCA
     240       250       260       270       280       290

            460       470       480       490       500       510
bFAA   CGACTATCAGGCTGGAGACGCAGATGTGTTTTCCAGGGAACCCTTTGTGGTCTGGTTCCA
        :::::::::::::::::::::::::: :::::::::: ::::::::: :::::::::
eFAA   TGACTATCAGGCTGGAGACGCAGATGTATTTTCCAGGGAGCCCTTTGTGATCTGGTTCCA
     300       310       320       330       340       350

            520       530       540       550       560       570
bFAA   GTCACCCTACACCGCTGTCAAGGACTTCGTGATTGTCCCCCTGCACACCACCCCTGAGAC
       :::::::::::::::::::::::::: :::::::::::::::::::::::::::::: ::
eFAA   GTCACCCTACACCGCTGTCAAGGACTTTGTGATTGTCCCCCTGCACACCACCCCTGATAC
     360       370       380       390       400       410

            580       590       600       610       620       630
bFAA   ATCCGTTAGAGAGATTGATGAGCTGGCTGATGTCTACACAGATGTGAAACGTCGCTGGAA
       :::::::: ::::::::::::::::::::::::::   :: :::::::: :: :::::
eFAA   ATCCGTTAAAGAGATTGATGAGCTGGCTGATGTCTACGTGGACGTGAAACGCCGTTGGAA
     420       430       440       450       460       470
```

```
            640       650       660       670       680       690
bFAA   TGCAGAGAATTTCATTTTCATGGGTGACTTCAATGCTGGCTGCAGCTACGTCCCCAAGAA
        :::::::::::::::::::::::::::::::: :: ::::::::::::::::::::::
eFAA   GGCAGAGAATTTCATTTTCATGGGTGACTTCAACGCCGGCTGCAGCTACGTCCCCAAGAA
     480       490       500       510       520       530

            700       710       720       730       740       750
bFAA   GGCCTGGAAGGACATCCGCCTGAGGACGGACCCCAAGTTCGTTTGGCTGATCGGGGACCA
       :::::::::: :::::::::::: :: ::::    ::: ::::::::::::::::  :
eFAA   GGCCTGGAAGAACATCCGCCTGAGAACCGACCTTGGGTTTGTTTGGCTGATCGGGGATGA
     540       550       560       570       580       590

            760       770       780       790       800       810
bFAA   AGAGGACACCACGGTCAAGAAGAGCACAAACTGCGCCTATGACAGGATCGTGCTTAGAGG
       :::::::::::::: ::::  ::::: :: :: :: :::::::::::: ::::::::::
eFAA   AGAGGACACCACGGTGAAGAGCAGCACCAAGTGTGCATATGACAGGATTGTGCTTAGAGG
     600       610       620       630       640       650

            820       830       840       850       860       870
bFAA   ACAAAATATTGTCAACTCTGGTGGTCCTCAATCAAACCTCGTCTTTGATTTCCAGAAAGC
       :: : : ::::::: ::::: :: :::  ::::::::  ::::::  : ::::::::: :
eFAA   ACGAGAGATTGTCAGCTCTGTTGTTCCCAAATCAAACAGCGTCTTCAACTTCCAGAAATC
     660       670       680       690       700       710

            880       890       900
bFAA   TTACAGGTTGTCTGAATCGAAGGCCCTGG
       :::    ::: :::::  : :::::::::
eFAA   TTATGCTTTGACTGAAGAGGAGGCCCTGG
     720       730       740       750
```

Figure 5.4. Comparison of translated nucleotide sequence of equine fertility-associated antigen (eFAA) to bovine FAA (bFAA) amino acid sequence. Identical residue matches are listed in the middle with plus (+) symbols representing a positive match. Absence of notation signifies no homology. A 243 amino acid residue overlap exists between bFAA and eFAA with 210 residues (or 86%) being identical. There are 226/243 residues (93%) that are positive or conserved amino acid substitutions. Note that most variation is apparent near the carboxyl terminus.

```
eFAA 19  HVRSFGESKKENRNTMDVIVKVIKRCDIILLMEIKDSNNMICPTLMEKLNGNSRRGITYN 198
         +VRSFGESKK N N MDVIVKVIKRCDIILLMEIKDS+N ICPTLMEKLNGNSR+GITYN
bFAA 27  NVRSFGESKKANCNAMDVIVKVIKRCDIILLMEIKDSSNRICPTLMEKLNGNSRKGITYN 86

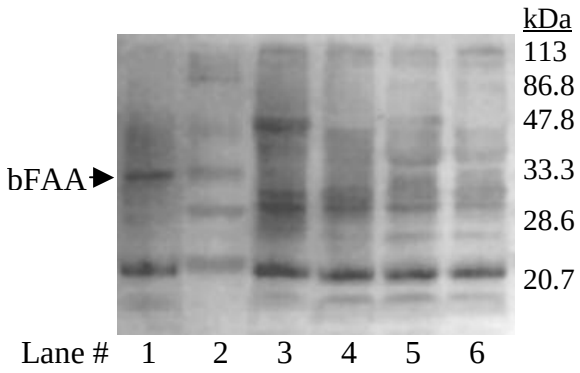
eFAA 199 YVISSRLGRNTYKEQYAFLYKEKLVSVKKRYLYHDYQAGDADVFSREPFVIWFQSPYTAV 378
         YVISSRLGRNTYKEQYAFLYKEKLVSVK+ YLYHDYQAGDADVFSREPFV+WFQSPYTAV
bFAA 87  YVISSRLGRNTYKEQYAFLYKEKLVSVKQSYLYHDYQAGDADVFSREPFVVWFQSPYTAV 146

eFAA 379 KDFVIVPLHTTPDTSVKEIDELADVYVDVKRRWKAENFIFMGDFNAGCSYVPKKAWKNIR 558
         KDFVIVPLHTTP+TSV+EIDELADVY DVKRRW AENFIFMGDFNAGCSYVPKKAWK+IR
bFAA 147 KDFVIVPLHTTPETSVREIDELADVYTDVKRRWNAENFIFMGDFNAGCSYVPKKAWKDIR 206

eFAA 559 LRTDLGFVWLIGDEEDTTVKSSTKCAYDRIVLRGREIVSSVVPKSNSVFNFQKSYALTEE 738
         LRTD  FVWLIGD+EDTTVK ST CAYDRIVLRG+ IV+S  P+SN VF+FQK+Y L+E
bFAA 207 LRTDPKFVWLIGDQEDTTVKKSTNCAYDRIVLRGQNIVNSGGPQSNLVFDFQKAYRLSES 266

eFAA 739 EAL 747
         +AL
bFAA 267 KAL 269
```

Figure 5.5. A tissue immunoblot was performed using anti-recombinant bovine fertility-associated antigen rabbit polyclonal antibody (rFAA Ab) to examine presence/absence of FAA in stallion tissue. Protein extracted from bovine tissue harvested from the seminal vesicles served as the immunoblot control following methods as described herein for stallion tissue. Each lane of a SDS-PAGE gel was loaded with 50 μg of total protein from each respective tissue source. After gel electrophoresis, protein was transferred on to nitrocellulose membrane and incubated with primary (rFAA Ab) and secondary antibodies and developed as described in Materials and Methods. Lane 1, bovine seminal vesicles; lane 2, protein standards, phosphorylase B (113 kDa), bovine serum albumin (86.8 kDa), ovalbumin (47.8 kDa), carbonic anhydrase (33.3 kDa), soybean trypsin inhibitor (28.6 kDa), lysozyme (20.7 kDa); lanes 3-6 tissue protein extracts from stallion liver, bulbourethral, prostate and seminal vesicular glands, respectively.



APPENDIX ONE

I. Characterization and Description of Recombinant Fertility-associated Antigen (rFAA) and Antibodies for FAA and TIMP-2.

Antisera used in experiments in this dissertation were produced at the University of Arizona (anti-rFAA), or were provided by Dr. W.G. Stetler-Stevenson (National Cancer Institute; Bethesda, MD) for TIMP-2 localization, or were purchased commercially (Amersham Biosciences, Piscataway, NJ) for the TIMP-2 ELISA using bovine seminal fluid.

A. Fertility-Associated Antigen (FAA)

The rabbit anti-rFAA polyclonal antisera were generated by immunizing rabbits with a recombinant preparation of FAA purified by HPLC as described (McCauley et al., 1999). That piece spanned amino acid residues 73 through 269 out of 296 predicted amino acids in the parent FAA (Zhang et al., 2003). The DNA construct was sequenced from 5' to 3' and 3' to 5' to confirm its orientation in the E. coli vector. The expressed protein was 22 kDa in size and was recognized by the monoclonal antibody M1, originally developed by Bellin et al. (1996) at the University of Arizona, in E. coli cell extracts (figure 6.1). After purification by reverse-phase HPLC and gel electrophoresis (figure 6.2a), that product was recognized as a single band on a Western blot using the M1 antibody (figure 6.2b) and was absent in the secondary control immunoblot (figure 6.2c). M1 cross-reactivity with a 31 kDa seminal protein formed the basis for

identification of FAA and led to the cloning and expression of recombinant FAA shown in Figure 6.2a.

The recombinant FAA fragment produced at the University of Arizona through funding by the USDA-SBIR grant was used by Strategic Biosolutions to immunize rabbits. The rabbit polyclonal anti-rFAA sera produced by this contractual service was then affinity purified across a column of TIMP-2 (a heparin-binding protein) to remove any sera that may recognize TIMP-2 in seminal components by Midland Bioproducts (Boone, IA). The use of the antisera herein was granted by ReproTec, Inc. The anti-rFAA recognized a specific band of the appropriate size by Western blot utilizing seminal fluid (figure 6.3a) and sperm-associated protein extracts (figure 6.4a) from species used in this dissertation. Pre-immune sera harvested from the same rabbits did not label the same protein that corresponded to FAA or any bands of the appropriate size by Western blotting (figure 6.3b and 6.4b). Moreover, that pre-immune sera did not label the surface of sperm when utilized as the appropriate control for immunolocalization experiments (Chapter 2).

In addition, the rFAA used for antibody development also contained biological activity. Recombinant FAA potentiated heparin-induced capacitation of bovine sperm *in vitro* at a dose of 200 ug/ml (Lenz et al., 2000). FAA shares homology to a DNase I-like protein (McCauley et al., 1999). Two DNase motifs are present within the rFAA fragment originally produced and used for immunization. Recombinant FAA (shown in 6.2a) nuclease activity was observed by adding increasing concentrations of rFAA to

purified plasmid DNA (figure 6.5a,b), demonstrating that rFAA was enzymatically active.

B. Tissue Inhibitor of Metalloproteinases-2 (TIMP-2)

Specificity of the anti-TIMP-2 rabbit antibody has previously been described by Stetler-Stevenson et al. (1990) and used for immunoblotting in ovine (Smith et al., 1995). TIMP-2 is also evolutionarily conserved between bovine and human as demonstrated by mRNA cross-hybridization studies (Zafarullah et al., 1996). Figure 6.8 illustrates the amino acid residue homology between bovine and human TIMP-2 with an overall identity of 94.3%. Utilizing the TIMP-2 antibody, as described in ovine (Smith et al., 1995), specific cross-reactivity was observed on the Western blot of sperm extracts from bull, ram and stallion at 21 kDa (figure 6.7a). The cross-reactivity was absent in the secondary alone control (figure 6.7b). Specificity of the TIMP-2 antisera was also described by immunoblot (Smith et al., 1995) and by the use of normal rabbit serum in immunolocalization experiments described in this dissertation (Chapter 2).

The monoclonal antibodies used in the TIMP-2 ELISA were developed from synthetic oligopeptides specific to the amino- and carboxyl-terminus of TIMP-2 (Fujimoto et al., 1993) based on the amino acid sequence first determined by Stetler-Stevenson et al. (1989). In addition, oligopeptides created by Fujimoto et al. (1993) spanned amino acid residues 30-44 and 178-193. Within those regions, bovine TIMP-2 is 100% identical to human TIMP-2 (figure 6.8). This antibody system had previously been validated and used to detect TIMP-2 in serum from mouse, rat, guinea pig, rabbit and bovine (Fujimoto et al., 1995).

C. Conclusion

In summary, specificity of the anti-rFAA antisera used for immunolocalization and quantification studies in this dissertation has been defined and characterized by 1) cross-reactivity against the immunogen (HPLC-purifed recombinant FAA); 2) cross-reactivity against native FAA from seminal plasma; 3) cross-reactivity against native FAA from sperm extracts; 4) lack of cross-reactivity with pre-immune sera in all of the above experiments; and 5) M1 mAb cross-reactivity against the same recombinant protein used for immunization to produce the anti-rFAA antisera. All of the described immunoblot data in this Appendix are supported at the molecular level by the presence within the FAA cDNA of three embedded peptides (deduced amino acid sequence) originally determined by micro-sequencing of native seminal FAA. That finding verified that the cloned FAA sequence used to produce the recombinant protein and the polyclonal antisera in question represented the authentic FAA gene.

Characterization of the TIMP-2 antisera is very well described in the literature, has been utilized by a number of laboratories and its specificity was verified by immunoblots in this Appendix. Interspecies identity of TIMP-2 amino acid sequence allowed anti-TIMP-2 peptide antisera to be produced and commercialized as reagents for the ELISA used in Chapter 3. Collectively, these reagents permitted the definitive determination of cellular localization and quantification of FAA and TIMP-2 on sperm and in seminal plasma, respectively.

Figure 6.1. Western blot using the monoclonal antibody (M1) on cell culture media (lane 1), whole cell extracts after induction (lane 2), supernatant extract after addition of cell lysis buffer (lane 3) and remaining protein in cell pellet (after lysis and supernatant removal; lane 4). The M1 antibody recognized a 22-kDa protein after cells were induced (lane 2). That product was present after cell lysis in supernatant (lane 3) and the cell pellet of the lysate.

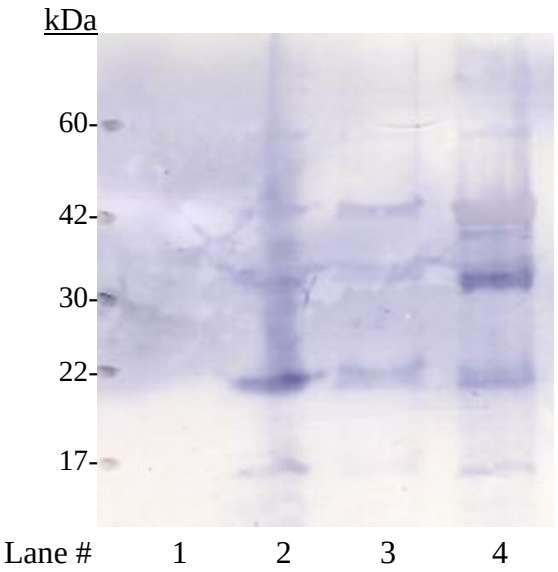


Figure 6.2. A.) SDS-PAGE of HPLC purified recombinant fertility-associated antigen (rFAA) and recognition by M1 and rFAA polyclonal antibody. 20 ug of HPLC purified FAA was loaded per lane. Lane 1, protein standards, phosphorylase B (142 kDa), bovine serum albumin (97.2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35.1 kDa), soybean trypsin inhibitor (29.7 kDa), lysozyme (21.9 kDa); lanes 2 and 3, rFAA. The rFAA HPLC purified fraction shown was used to immunize rabbits for the development of rFAA polyclonal antibodies. B.) Lanes 1 and 2, Western blot of rFAA using the original monoclonal antibody (M1; Bellin et al., 1996). C.) Lanes 1 and 2, secondary antibody alone (control) for M1. D.) Lanes 1 and 2, Western blot using polyclonal rFAA antibody to rFAA immunogen (shown in A). The colorimetric reaction was allowed to proceed for 3 min. E.) Lanes 1 and 2, pre-immune sera from the same rabbit (control for D) to rFAA immunogen (shown in A).

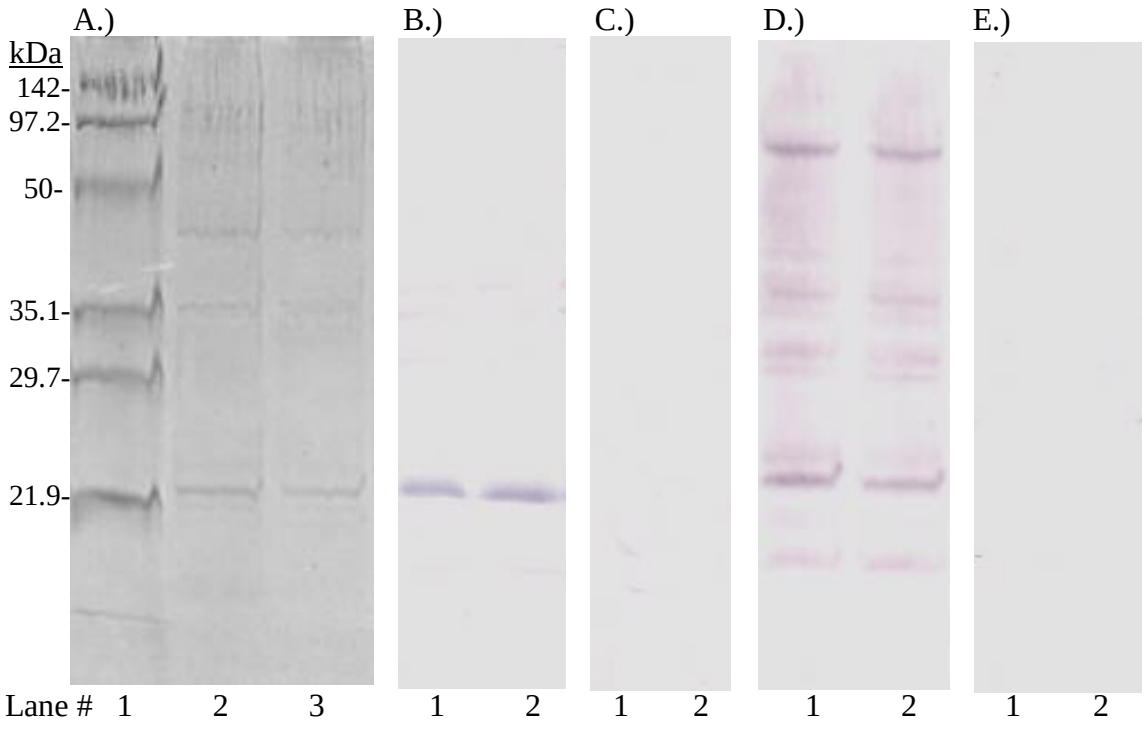


Figure 6.3. A.) Western blot of seminal plasma from bull (lane 1 and 2), ram (lane 4) and boar (lane 5) using anti-recombinant FAA polyclonal serum (1:1000). Lane 3, protein standards, phosphorylase B (142 kDa), bovine serum albumin (97.2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35.1 kDa), soybean trypsin inhibitor (29.7 kDa), lysozyme (21.9 kDa). B.) Pre-immune rabbit sera (1:1000) was used as the control for all species (lane 6, blank; lane 7 and 8 bull; lane 9, ram; and lane 10, boar.

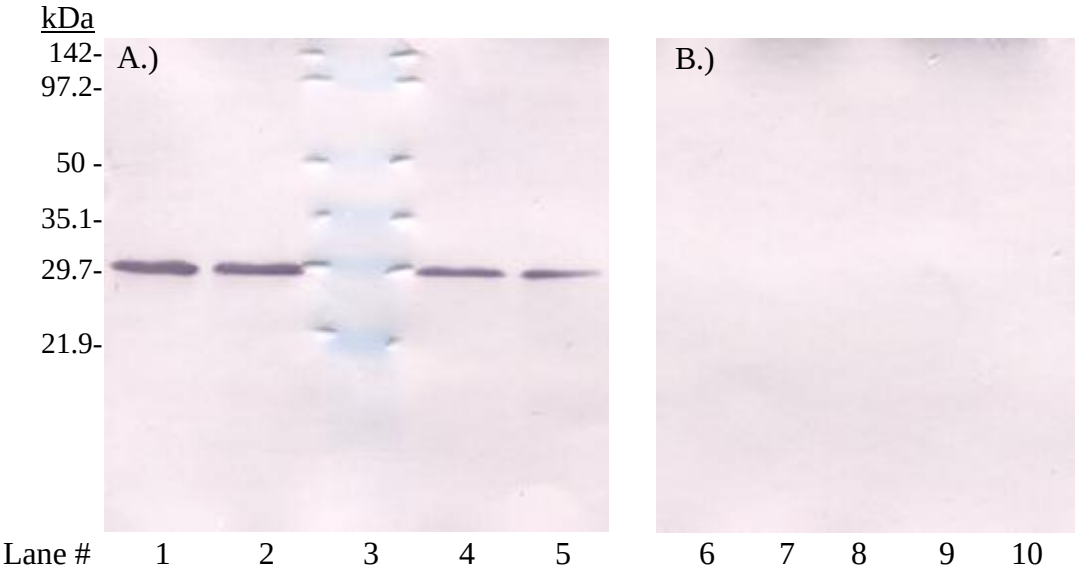


Figure 6.4. A.) Western blot of sperm extracts from bull (lanes 1 and 2), ram (lane 3), and boar (lane 5) using anti-recombinant FAA polyclonal serum (1:1000). Lane 4, protein standards, phosphorylase B (142 kDa), bovine serum albumin (97.2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35.1 kDa), soybean trypsin inhibitor (29.7 kDa), lysozyme (21.9 kDa). B.) Pre-immune rabbit sera (1:1000) was used as the control for all species (lane 6 and 7, bull; lane 8, ram; and lane 9, boar sperm.

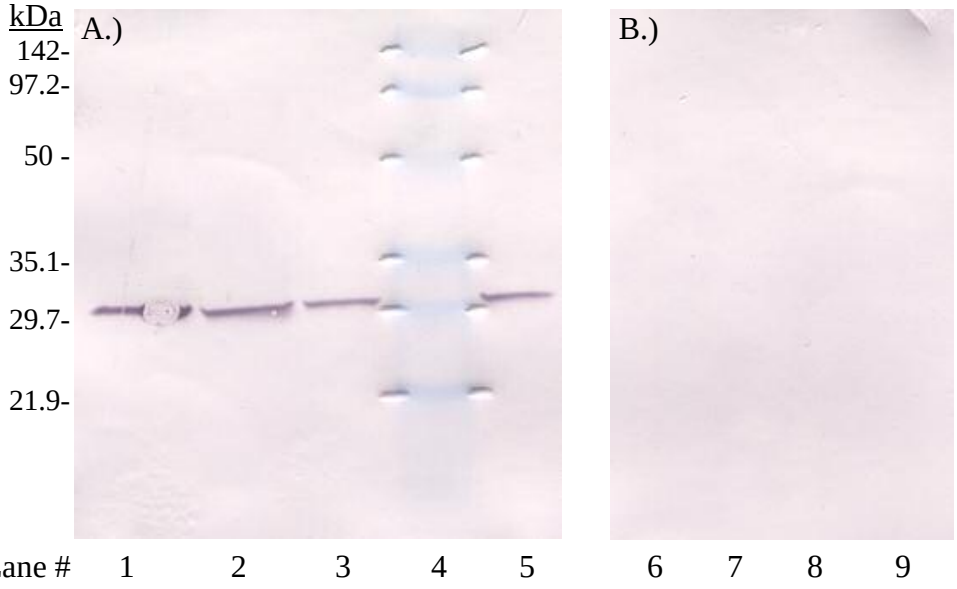


Figure 6.5. DNase activity of HPLC purified recombinant FAA (shown in 6.1a) as visualized by agarose gel electrophoresis. A.) Control plasmid (CP) DNA (1.0 ug) was digested using increasing concentrations from 0.10 to 5.0 ug/ml of rFAA, and B.) 7.5 to 25.0 ug/ml of rFAA. Incubations were performed for 1-h at room temperature using nuclease free water in the absence of $MgCl_2$. CP in the absence of rFAA served as the control. Nuclease activity was observed at concentrations $\geq$ 5.0 ug/ml of rFAA. A 100 bp DNA molecular ruler (MR) is provided for reference.

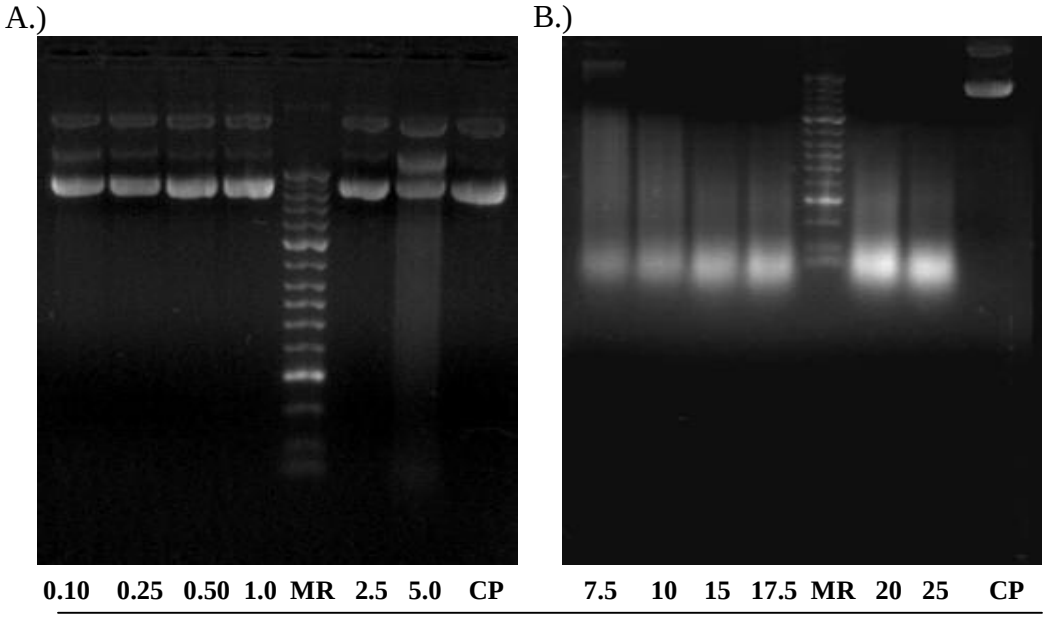


Figure 6.6. A.) Western blot of sperm extracts from bull (lane 1), ram (lane 3), boar (lane 4) and stallion (lane 5) using anti-tissue inhibitor of metalloproteinases-2 (TIMP-2) polyclonal serum (1:750). Gel electrophoresis was performed under non-reducing conditions. Lane 2, protein standards, phosphorylase B (142 kDa), bovine serum albumin (97.2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35.1 kDa), soybean trypsin inhibitor (29.7 kDa), lysozyme (21.9 kDa). B.) Secondary anti-rabbit antibody (1:20,000) alone served as the control for all species (lane 6, bull; lane 7, ram; lane 8, boar; and lane 9, stallion).

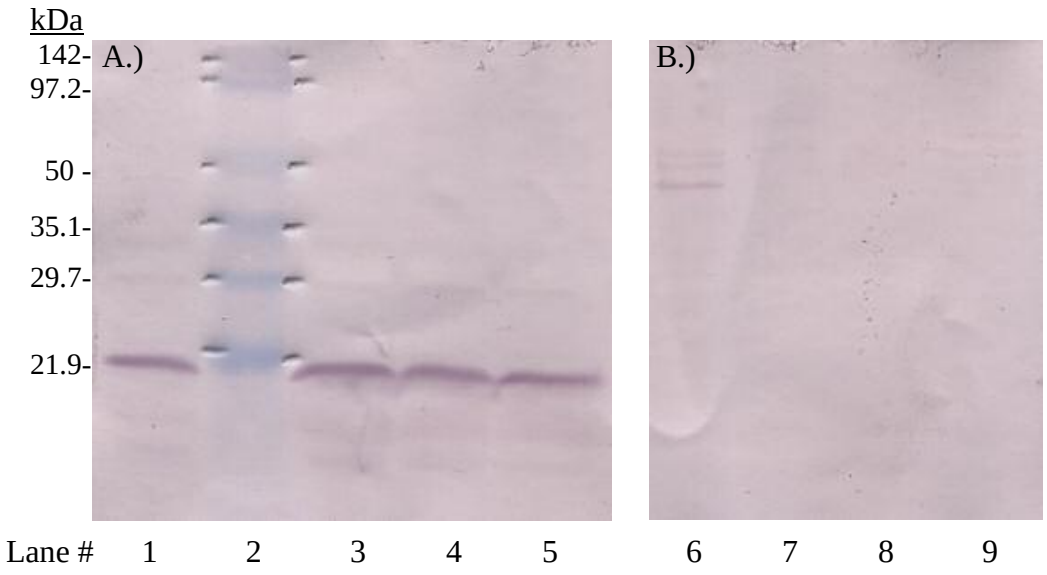


Figure 6.7. Multi-alignment of human TIMP-2 (hTIMP-2; accession number NP_003246) and bovine TIMP-2 (bTIMP-2; accession number NP_776897). Oligopeptides which were used to create the monoclonal antibodies for the Biotrak TIMP-2 ELISA span residues 30-44 and 178-193 (Fujimoto et al., 1993; 1995). Those regions are 100% identical between human and bovine (see boxed regions below). The overall homology between the human and bovine TIMP-2 protein is 94.3%.

```
                10        20        30        40        50        60
hTIMP-2  CSCSPVHPQQAFCNADVVIRAKAVSEKEVDSGNDIYGNPIKRIQYEIKQIKMFKGPEKDI
         :::::::::::::::::.::::::..:::::::::::::::::::::::::::::..::
bTIMP-2  CSCSPVHPQQAFCNADIVIRAKAVNKKEVDSGNDIYGNPIKRIQYEIKQIKMFKGPDQDI
                10        20        30        40        50        60

                70        80        90       100       110       120
hTIMP-2  EFIYTAPSSAVCGVSLDVGGKKEYLIAGKAEGDGKMHITLCDFIVPWDTLSTTQKKSLNH
         :::::::..:::::::::::::::::::::::.:::::::::::::::::::.::::::::
bTIMP-2  EFIYTAPAAAVCGVSLDIGGKKEYLIAGKAEGNGNMHITLCDFIVPWDTLSATQKKSLNH
                70        80        90       100       110       120

               130       140       150       160       170       180
hTIMP-2  RYQMGCECKITRCPMIPCYISSPDECLWMDWVTEKNINGHQAKFFACIKRSDGSCAWYRG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
bTIMP-2  RYQMGCECKITRCPMIPCYISSPDECLWMDWVTEKNINGHQAKFFACIKRSDGSCAWYRG
               130       140       150       160       170       180

               190
hTIMP-2  AAPPKQEFLDIEDP
         ::::::::::::::
bTIMP-2  AAPPKQEFLDIEDP
               190
```

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