Journal of Andrology, Vol. 27, No. 2, March/April 2006

Identification of Proteins in the Accessory Sex Gland Fluid Associated With Fertility Indexes of Dairy Bulls: A Proteomic Approach

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ABSTRACT: We evaluated the expression of proteins in the accessory sex gland fluid (AGF) and their relationships with fertility indexes of dairy bulls. Fertility was normalized as the percentage point deviation of their nonreturn rates (PD) from the average fertility of all bulls from a given artificial insemination center. Services associated with each sire ranged from 269 to 77 321 and PD values from +7.7% to −18.1%. AGF, from 37 bulls, was obtained with an artificial vagina after cannulation of the vasa deferentia. Proteins from AGF were separated by 2-dimensional SDS-PAGE followed by staining with Coomassie blue and analysis of polypeptide maps using PDQuest software. Bulls were divided in groups based on PD values and the optical density of spots in the AGF gels used as independent variables to predict bull fertility. Proteins were identified by capillary liquid chromatography nanoelectrospray ionization tandem mass spectrometry (CapLC-MS/MS). An average of 52 ± 5 spots was detected in the AGF gels, but there were no spots unique to groups of either high- (PD ≥ 0) or low- (PD < 0) fertility sires. The former were neither less nor more homogeneous than the latter based on correlations of all matched spots between pairs of AGF maps. However, high fertility of dairy bulls was significantly associated with lower expression of 14-kDa spermadhesin Z13 isoforms and higher amounts of 55-kDa osteopontin and 58-kDa phospholipase A_2 (PLA_2) isoforms. The average intensity of 5 spots identified as BSP 30 kDa in the AGF gels had a quadratic association with fertility indexes ($R^2 = .18$; $P = .03$). PD values of bulls were related ($R^2 = .56$) to the quantity of spermadhesin, osteopontin, and BSP 30 kDa in the AGF polypeptide maps. Bull fertility was also determined by another equation ($R^2 = .53$) with spermadhesin, BSP 30 kDa, and PLA_2 as independent variables. We conclude that interactions among several proteins in accessory sex gland fluid explain a significant proportion of the variation in fertility scores of mature dairy sires.

Key words: BSP 30 kDa, osteopontin, phospholipase A_2, seminal plasma, spermadhesin.

J Androl 2006;27:201–211

Secretions from the accessory sex glands are mixed with sperm at ejaculation and contribute to the majority of semen volume and components. Some accessory sex gland (AG) proteins are known to bind to the spermatozoa membrane and affect its function and properties (Yanagimachi, 1994), and depletion of those glands in mice causes reduction in embryonic development and numbers (Chen et al, 2002), suggesting that components of the AG may have a paternal influence on postfertilization events. From another prospective, fluid collected from the accessory sex glands of bulls has been shown to enhance the oocyte-penetrating capacity of sperm from the cauda epididymis (Henault et al, 1995). Taken together, this evidence supports the notion that accessory sex gland secretions participate in key events related to sperm function, fertilization, and embryo development in the female reproductive tract. However, despite these observations, evidence linking AG proteins with fertility indexes has been a major challenge. Few quantitative measurements of those potential molecular markers exist in males for which reliable data sets also exist for reproductive performance. In the case of dairy sires, data sets are available to assess fertility of individual males based on insemination of large numbers of cows using semen that has been frozen by standardized procedures. This information has been used to demonstrate that fertility-associated proteins exist in bull seminal plasma (Killian et al, 1993). However, because seminal plasma is comprised of secretions originating from the accessory sex glands, epididymis, and the testis, it is difficult to define the contributions of these organs to reproductive performance.

Previously, we utilized a technique to obtain secretions from the accessory sex glands and cauda epididymis by catheterization of the vas deferens (Henault et al, 1995). This unique model enables the collection of accessory

Supported by grants 2003-34437-13460 and 2004-34437-15106 from the US Department of Agriculture.

†Supported by a Fellowship awarded by the Brazilian Research Council (CAPES).

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Received for publication May 5, 2005; accepted for publication August 2, 2005.

DOI: 10.2164/jandrol.05089

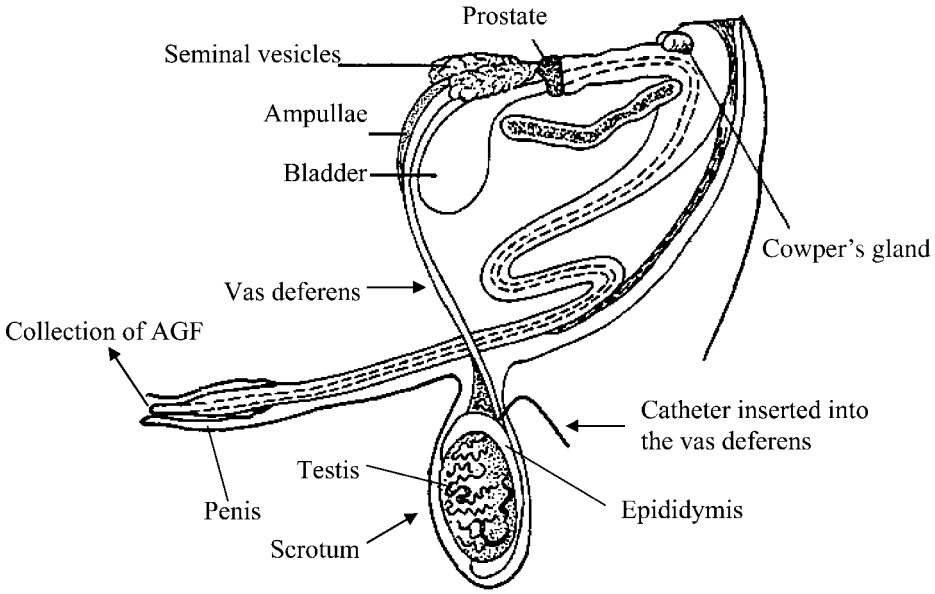

Figure 1. Diagram of the bull reproductive tract (adapted from Salisbury et al, 1978) showing the strategy for collection of accessory sex gland fluid (AGF). As described by Henault et al (1995), a polyethylene catheter was used to connect the vas deferens. The catheter was inserted in a Dracon felt disk and glued with medical-grade silastic adhesive and the disk, placed subcutaneously where the catheter exited the scrotum posteriorly. Fluid from accessory sex glands was collected by an artificial vagina, free of contact with epididymal secretions and sperm.

gland fluid from living individuals, free of germ cells and components from the testis and epididymis. Although the surgically altered animal does not perfectly mimic the events that occur naturally, when secretions from accessory sex glands are mixed with spermatozoa and epididymal fluid at ejaculation, this model does make it possible to obtain for study a composite of accessory gland secretions mixed by the living animal. In the current study, we report the use of a proteomic approach to identify proteins in samples of accessory sex gland fluids from Holstein bulls of documented fertility and determine if relationships exist between their expression and fertility indexes.

Materials and Methods

Experimental Design

Accessory sex gland fluid (AGF) from mature Holstein bulls of known fertility was subjected to bidimensional sodium dodecyl sulfate-polyacrilamide gel electrophoresis, and gel images were analyzed by a computerized system. We determined the relationships between protein intensity in the 2-dimensional polypeptide maps and fertility scores of the bulls, and proteins were identified by tandem mass spectrometry. Samples of AGF were obtained from 37 animals that had been surgically altered by inserting indwelling catheters in the vas deferens, which allowed the collection of AGF without sperm or secretions from the epididymis (Henault et al, 1995; Figure 1). Fluid from the accessory sex glands was collected with an artificial vagina, centrifuged at 2400 $\times$ *g* for 15 minutes, and stored in liquid nitrogen for later analysis. This project was approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University.

Fertility Indexes of Bulls

Holstein bulls and information about nonreturn rates (NRRs) were provided by artificial insemination (AI) cooperatives in the northeastern United States. The number of services using frozen semen from each bull ranged from 269 to 77 321. Individual NRRs for each bull were based on the number of cows that did not return to be inseminated within 60 days after the first insemination, relative to the total number of cows that were inseminated. To compensate for small variations among data sets obtained from different AI centers, the fertility index of each bull was expressed as the percentage point deviation (PD) of its NRR from the average NRR of all bulls in a given AI center (Killian et al, 1993). Bulls used in the present study had PDs from the average of +7.7% to −18.1%. There were 18 animals with PD $\geq$ 0 and 19 with PD $<$ 0.

Electrophoresis

Samples of AGF were removed from liquid nitrogen, thawed at room temperature, and centrifuged at 10 000 $\times$ *g* (60 minutes at 5°C). The supernatant was then assayed for protein content (Lowry et al, 1951) using bovine serum albumin as standards and aliquots, frozen at −80°C. When used for electrophoresis, samples were thawed at room temperature and subjected to 2-dimensional electrophoresis (Killian et al, 1993). Isoelectric focusing was carried out in tube gels (Bio Rad, Rockville Centre, NY) containing a mixture of ampholytes with pH 3–7 (0.4 ml) and 3–10 (0.1 ml; Serva, Heideberg, Germany). Samples of AGF containing 500 μg of protein were brought to a volume of 100 μL using a solution of β-mercaptoethanol, urea, and the same

ampholytes used in the gels. Gels were then subjected to 200 V for 15 minutes, 300 V for 30 minutes, 400 V for 30 minutes, 375 V for 16–18 hours, and 800 V for 1 hour. Following focusing, gels were removed from the tubes and placed on stacking gels that had been prepared on the top of gels containing a linear gradient of acrylamide (10%–17.5%). Standards from 66 to 14 kDa were also used (Sigma Chemical Co, St Louis, Mo). Gels were stained with Coomassie brilliant blue R-250; destained in a solution of methanol, acetic acid, and deionized distilled H_20; and scanned using a GS-670 imaging densitometer (Bio Rad, Rockville Centre, NY). Images saved as TIFII files were analyzed using PDQuest software, version 7.3.0 (Bio Rad). For the set of AGF polypeptide maps, a single master gel was generated by the software, which represented the best pattern of spots in the samples. Few additional spots consistently present in some gels were also added to the master so that they could be matched to all samples. Proteins in key regions of the gels were used as landmarks and final matching of spots was achieved after several rounds of extensive comparisons. Control of spot matches was done by checking each spot in each gel with the respective pattern in the master. Protein quantities in the gels were given as ppm of the total integrated optical density of the spots, according to PDQuest.

Preliminary results revealed the presence of an unidentified low–molecular-weight polypeptide in the AGF that was inversely related to bull fertility scores. The molecular weight and pI for this peptide were similar to those of a low-fertility protein (~16 kDa; pI 6.7) originally described by Killian et al (1993) in the seminal plasma of another group of Holstein bulls. Therefore, to determine if these proteins shared the same identity, seminal plasma from bulls used in Killian's original study was thawed after storage in a −80°C freezer, subjected to bidimensional electrophoresis, and three spots at ~14 kDa and pIs 6.7–6.9 were analyzed by tandem mass spectrometry.

Protein Identification

Proteins separated by 2-dimensional SDS-PAGE and selected by PDQuest software (Bio Rad) were subjected to in-gel trypsin digestion as described elsewhere (Koc et al, 2001). Excised gel pieces were washed three times with 100 μL of ammonium bicarbonate (25 mM) and dehydrated with 100 μL of acetonitrile (50%), and dried in a speed vacuum. They were then incubated overnight at 37°C with trypsin (12.5 ng/μL in 25 mM ammonium bicarbonate). Peptides were then extracted twice with 25 μL of formic acid (5%) for 20 minutes. The extracts were dried in a speed vacuum again and resuspended in 10 μL of 5% acetonitrile with formic acid (0.1%).

Tryptic digests were analyzed by capillary liquid chromatography-nanoelectrospray ionization-tandem mass spectrometry (CapLC-MS/MS). A Micromass Q-Tof API US mass spectrometer coupled with a Waters CapLC high-performance liquid chromatography (HPLC) unit (Waters Co, Milford, Mass) was used for the analysis (Abbas et al, 2005). The proteolytic digests (1–5 μL) were injected into solvent A (acetonitrile/water/formic acid, 5/95/0.1) supplied by the auxiliary pump of the capillary HPLC unit and trapped in a Waters Symmetry 300 column (C-18, 5 μm film; 0.3 mm × 5 mm) for on-line desalting and preconcentration (Abbas et al, 2005). After washing for 3 minutes with solvent A at 20 μL/min, trapped peptides were then back flushed with the gradient solvent flow on to the analytical column, a Dionex PepMap fused-silica capillary column (C-18 5 μm, 0.075 mm × 150 mm), using a 10-port switching valve. The analytical column was run with a gradient (5%–42% solvent B; acetonitrile/water/formic acid; 95/5/0.2) in 44 minutes. The mass spectrometry was calibrated using Glu-Fib product ion fragments as needed to maintain mass accuracy within 10 ppm. The Q-Tof mass spectrometer was operated to acquire MS/MS of tryptic peptides in data-dependent acquisition mode for precursor ion selection using charge-state recognition and intensity threshold as selection criteria using MassLynx 4.0 SP1. In order to carry out the tandem mass spectrometric data acquisition, a survey scan (2 seconds) over the *m*/*z* of 400–1500 was performed. From each survey scan, up to 4 most intense precursor ions based on the selection criteria were selected for MS/MS to obtain the production spectra resulting from collision-induced dissociation in the presence of argon. The product ion spectra (6–8 seconds) collected were processed using Protein Lynx Global Server 2.1 and were converted to peak list text files for database searching. In order to identify the proteins, MS/MS ion searches were performed on the processed spectra against a locally maintained copy of the NCBI NR database using MASCOT Daemon and search engine (Matrix Science, Inc, Boston, Mass). The searches were made with the assumption that there was 1 maximum missed trypsin cleavage and that peptides were monoisotropic and oxidized at methionine residues and carbamidomethylated at cysteine residues. Peptide mass tolerance and fragment mass tolerance were initially set to 1.2 and 0.6 Da, respectively, for MS/MS ion searching. However, peptide mass values were ensured to be within 0.1 Da (typically less than 0.05 Da) when manually reviewing MASCOT search results.

Statistical Analysis

Bulls were divided in 4 groups based on fertility scores (PD values): group I (n = 6): $-18.1 \leq PD \leq -6.2$; group II (n = 13): $-5.9 \leq PD \leq -0.8$; group III (n = 12): $0.0 \leq PD \leq 2.6$; group IV (n = 6): $2.9 \leq PD \leq 7.7$. Differences in protein expression among these groups were evaluated by Duncan statistical test (SAS, 2003). Protein quantities (estimated by PDQuest) that significantly differed among bulls were used as independent variables in regression models (SAS, 2003) to predict the percentage PD of bull NNR.

Results

An average of 52 ± 5 spots was detected in the AGF bidimensional gels stained with Coomassie blue (Figure 2), and there were 19 spots detected in every single member of the match set constructed by PDQuest. There were no spots unique to any groups of bulls with different fertility categories. Correlations between intensities of all matched spots in pairs of gels (Figure 3) were similar, within a subset of 19 bulls with $PD \geq 0$ (168 comparisons; average $r = .84$), 18 bulls with $PD < 0$ (190 comparisons; average $r = .86$) and pairs of high and low

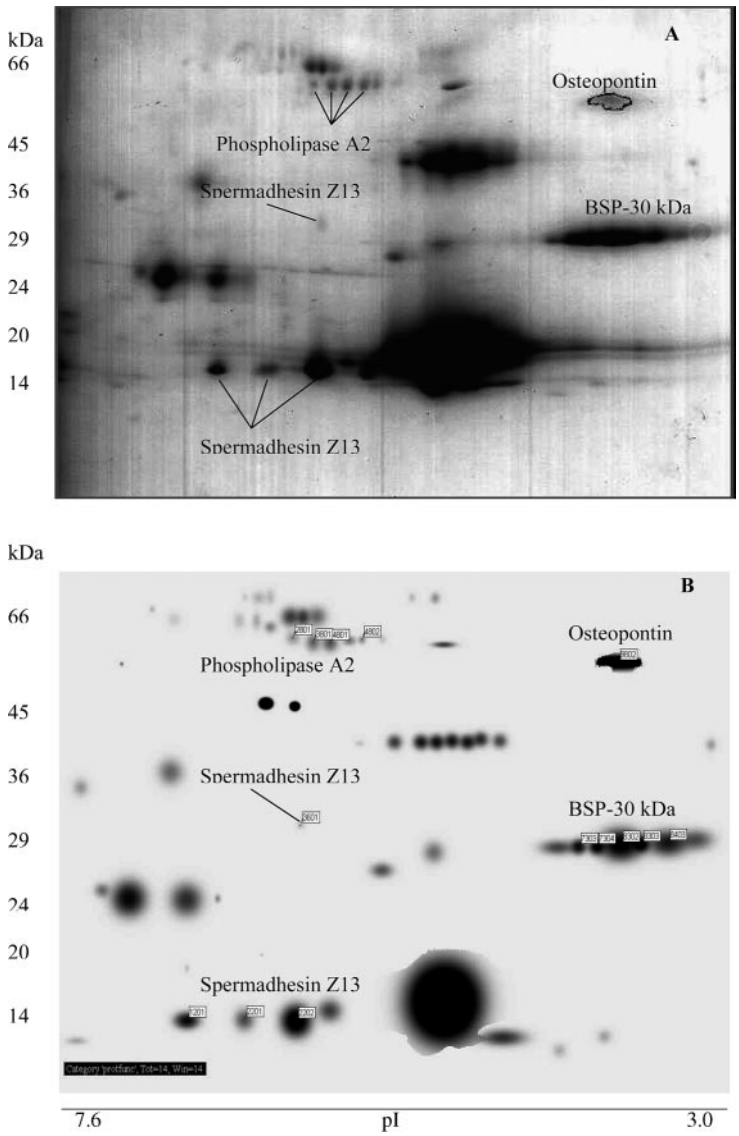

Figure 2. Protein profile of the bull accessory gland fluid obtained by 2-dimensional polyacrilamide gel electrophoresis (top) and the master gel constructed by PDQuest software (button). Intensity of spots identified as spermadhesin Z13 (average of spots 2201 and 2202), osteopontin (spot, 9802), BSP 30 kDa (average of spots 7303, 7304, 8302, 8303, and 8403), and phospholipase A_2 (average of spots 3801 and 4801) were significantly related to bull fertility. Proteins were identified by tandem mass spectrometry (CapLC-MS/MS).

fertility sires (342 comparisons; average $r = .85$). Correlations of matched spots in pairs of AGF 2-dimensional maps also had similar patterns using bulls from groups I ($-18.1 \leq PD \leq -6.2$), II ($-5.9 \leq PD \leq -0.8$), III ($0.0 \leq PD \leq 2.6$), and IV ($2.9 \leq PD \leq 7.7$).

The percentage PD of the average NRR of sires was significantly associated with the amount of 4 proteins identified in the 2-dimensional maps of accessory sex gland fluid. The average intensity of two 14-kDa spermadhesins (spots 2202 and 2201; Figure 2) was higher in bulls of low fertility (Figure 4). Sires with fertility scores between −18.1 and −6.2 had 2.2 times more spermadhesin than did bulls with fertility scores from 2.9 to 7.7 ($P < .05$). Both spermadhesin isoforms (spots 2202 and 2201) showed practically the same type of association with fertility. The spots excised from the seminal plasma gels (spots 1, 2, and 3; Figure 5) also matched to spermadhesin Z13 and had similar pI values as the ones present in the AGF. The most basic isoform of AGF (spot 1201; Figure 2) is equivalent to the one originally found as an antifertility factor in the seminal plasma (spot 1; Figure 5). Information about protein identification by MS/MS is presented in Table 1.

Bulls with the highest fertility scores ($2.9 \leq PD \leq 7.7$) had 2.3 times ($P < .05$) more of a 55-kDa osteopontin (spot 9802; Figure 2) than bulls with above-average fertility ($0.0 \leq PD \leq 2.6$) and at least 4 times ($P < .05$) more than bulls with below average ($-5.9 \leq PD \leq -0.8$)

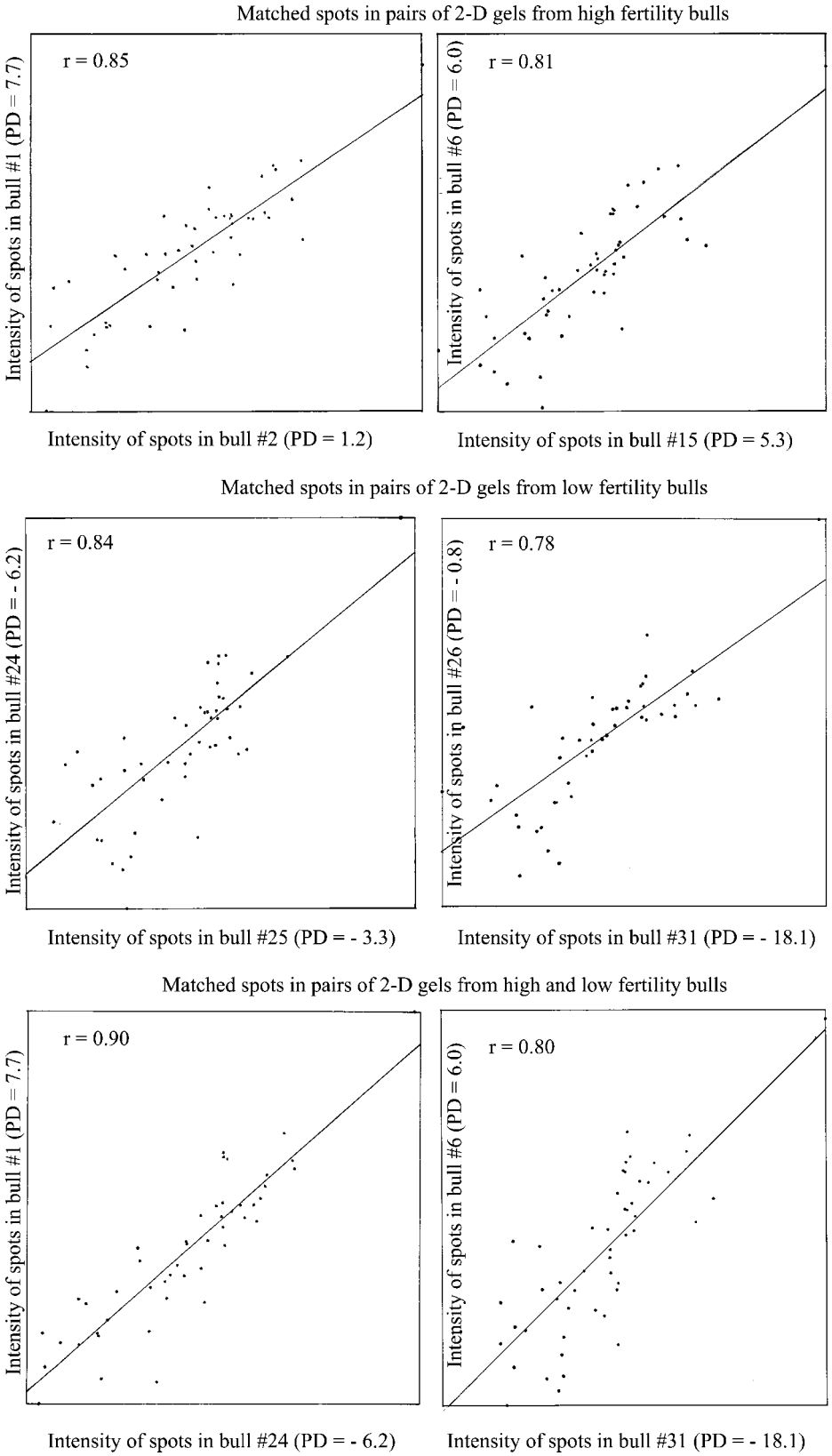

Figure 3. Correlations between the intensities of matched spots in pairs of accessory sex gland fluid 2-dimensional gels within groups of bulls with high- and low-fertility scores (percentage point deviation of nonreturn rate values). Spot intensities were estimated as PPM of the total integrated density of the spots. Graphs and *r* values were obtained from PDQuest software.

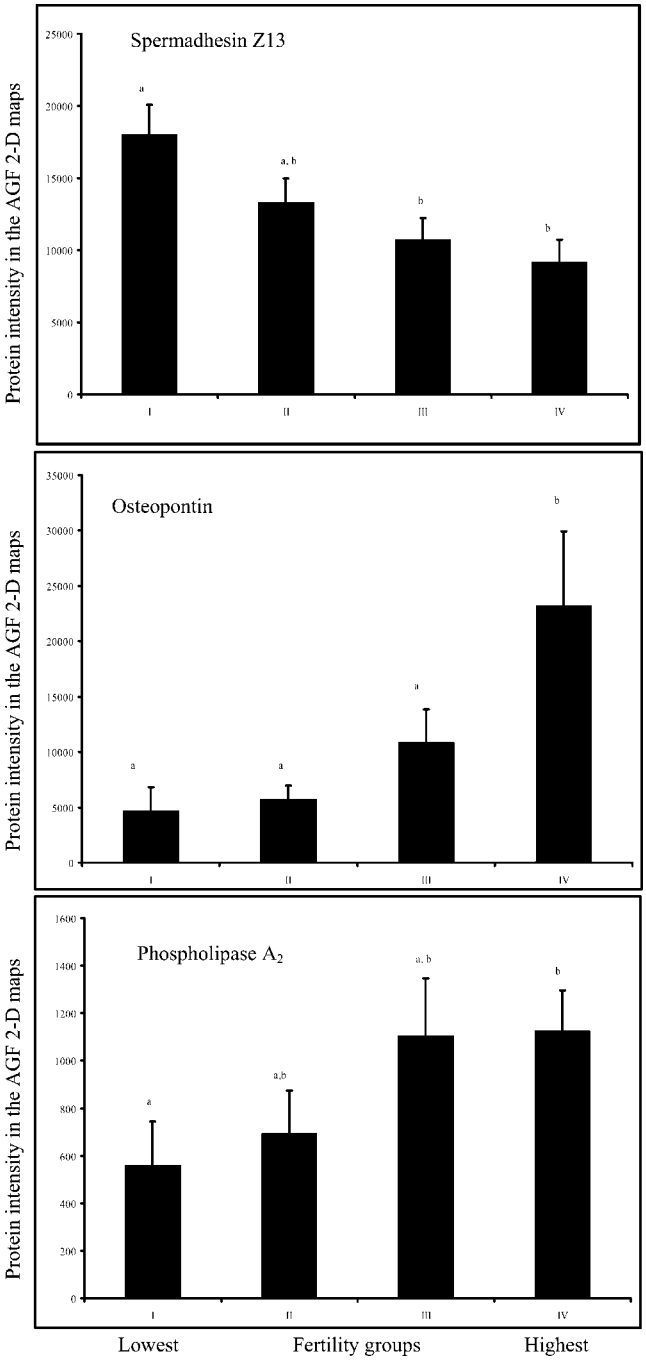

Figure 4. Intensity (and SE) of proteins identified as spermadhesin Z13, osteopontin, and phospholipase A_2 in 2-dimensional maps of accessory sex gland fluid (AGF) from Holstein sires. Bulls were grouped based on the percentage point deviation of their nonreturn rates (PD). Group I (n = 6): $-18.1 \leq PD \leq -6.2$; group II (n = 13): $-5.9 \leq PD \leq -0.8$; group III (n = 12): $0.0 \leq PD \leq 2.6$; group IV (n = 6): $2.9 \leq PD \leq 7.7$. Values followed by the same letters are not significantly different ($P > .05$).

or with the lowest fertility indexes of all ($-18.1 \leq PD \leq -6.2$; Figure 4). Intensity of two 58-kDa spots identified as phospholipase A_2 (spots 3801 and 4801; Figure 2) also showed variations among groups of bulls with different fertility (Figure 4), although changes were less pronounced than those observed with spermadhesin and osteopontin. Phospholipase A_2 (PLA_2) expression was similar in bulls with the highest ($2.9 \leq PD \leq 7.7$) and above-average fertility ($0 \leq PD \leq 2.6$) and significantly different ($P < .05$) only when compared with bulls with the lowest scores ($-18.1 \leq PD \leq -6.2$; Figure 4). These 2 spots were related to fertility in a similar manner and were part of a series of 4 PLA_2 isoforms identified by CapLC-MS/MS (Figure 2; Table 1). Some of the spots identified as BSP 30 kDa had a tendency to show either a negative or positive association with fertility, but none of these associations was significant. However, the average intensity of 5 BSP 30 kDa isoforms (spots 7303–8403; Figure 1) had a quadratic association with fertility indexes ($R^2 = .18$; $P = .03$), as shown in the plot of Figure 6.

Given the variations of those 4 AGF proteins among bulls of known fertility, regression models were constructed using the intensity of their respective spots as independent variables. An equation that included spermadhesin isoforms, osteopontin, and BSP 30 kDa isoforms explained a significant proportion ($R^2 = .56$; Table 2) of the variation in fertility scores. Bull fertility index was also related to the amount of 2 spermadhesins, BSP 30 kDa and PLA_2 isoforms ($R^2 = .53$, Table 2). A regression equation that included spermadhesin (average of 2 isoforms) as the single independent variable gave an R^2 of .33, and models with spermadhesin and osteopontin only or spermadhesin and BSP 30 kDa only gave R^2 values of .46 and .47, respectively.

Discussion

The use of 2-dimensional electrophoresis, computerized image analysis, and MS/MS has enabled us to compile protein profiles of the accessory sex gland secretions of high-use dairy bulls and relate them to a fertility phenotype. This is the first study to confirm that multiple proteins from the accessory sex gland fluid collected from the whole animal are associated with reproductive performance. Previous studies have shown similar associations between NRR and bovine seminal plasma proteins (Killian et al, 1993; Bellin et al, 1998). However, seminal plasma is a composite of secretions produced by testis, epididymis, and accessory sex glands, and protein interactions and biochemical modifications may affect the components originally secreted. The ability to analyze the components secreted exclusively from the accessory sex glands has provided unique information about proteins they secrete and that are correlated with fertility indexes. Relating expression levels of specific proteins to fertility phenotype should serve as a sound foundation to evaluate their role in sperm function and fertilization.

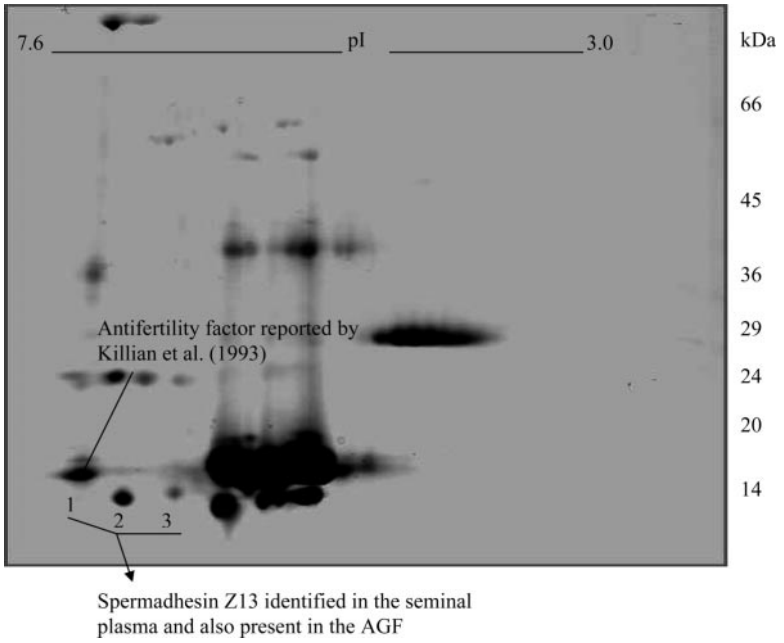

Figure 5. Profile of bull seminal plasma proteins obtained by 2-dimensional polyacrilamide gel electrophoresis. A basic low–molecular-weight antifertility factor reported by Killian et al (1993) was identified by tandem mass spectrometry in the present study as spermadhesin Z13. This same protein was also detected in the AGF and inversely related to fertility scores of bulls.

Only 40.4% of the spots detected in the AGF maps were found in all samples. Despite the degree of variation in the number of spots among gels, we were unable to identify any protein that was expressed exclusively in either high-fertility or low-fertility bulls, even when we compared sires with the highest fertility (PD values from +2.9 to +7.7) with those with the lowest scores (PD values from −6.2 to −18.1). High-fertility bulls were neither less nor more homogeneous than low-fertility bulls based on correlations of all matched spots between pairs of AGF gels.

Bulls used in this study were reproductively normal, with semen parameters that were uniformly satisfactory for the AI industry. Although the sires studied were not selected for specific reproductive traits, they represent a population of bulls that possess acceptable fertility and good semen qualities and freezing characteristics as young sires. Dairy bulls not meeting these criteria are culled early in reproductive life. Thus, differences in fertility scores among proven dairy sires do not represent the full range of fertilities seen in an unselected population and could not be related to a large number of peptides expressed in the AGF. Consequently, proven dairy sires may not exhibit fertility-associated peptides that are expressed in the fluids from the accessory sex glands of males with extremely low fertility.

The expression of 4 AGF proteins had distinct patterns among groups of sires with different fertility, and empirical regression models were useful in explaining the significance of those proteins for prediction of fertility. In one model, we obtained an R^2 of .56 based on the amount of 2 spermadhesins, osteopontin and isoforms of BSP 30 kD, and another regression model was obtained with a similar R^2 (.53) when the intensity of spots identified as PLA_2 were used as independent variables, along with that of the spermadhesins and BSP 30 kDa. Spermadhesin Z13 was the single factor that accounted for the greatest proportion of the variation in bull fertility (R^2 = .33). A combination of this peptide with osteopontin or BSP 30 kDa gave almost identical R^2 values (.46 and .47), suggesting that the contribution of PLA_2 for explanation of fertility measured in vivo was small, although significant.

The intensity of spermadhesin Z13 in AGF showed an inverse relationship with fertility. Consistent with these observations, the low–molecular-weight antifertility peptide reported by Killian et al (1993) in the seminal plasma of another group of Holstein bulls was also identified as spermadhesin Z13. The isoform originally described in the seminal plasma by those authors appears to be more basic (pI 6.7) than the ones we found in the AGF as antifertility factors (pI 6.5 and 6.3). Aspects of the methodology used for identification and quantification of spot intensity could have accounted for those subtle differences. Also, alterations may occur when accessory sex gland proteins are mixed with other components of the seminal plasma. According to Tedeschi et al (2000), spermadhesin Z13 appears as two 13-kDa monomers in 2-dimensional gels of bovine seminal plasma, which are believed to originate from a 26-kDa dimer. Similarly, we identified a spermadhesin Z13 spot in the AGF gels at 29 kDa.

Table 1. *Accessory sex gland fluid and seminal plasma proteins identified by 2-dimensional gel electrophoresis and capillary liquid chromatography nanoelectrospray ionization tandem mass spectrometry (CapLC-MS/MS)*

Protein Identification and Spot Number*	MW; pI*	Protein ID Code†	Sequence Coverage (%)	Matched Peptides
Osteopontin (spot 9802; from AGF)	55 kDa; 5.0	gi19774215	35	ISHELDASASEVN YPDAVATWLKPDPSQK LSQEFHSLEDKLDLDHK QTFLAPPQNSVSSEETDDNK QTFLAPQNSVSSEETDDNKQNTLPSK SNVQSPDATEEDFTSHIESEEMHDAPK
BSP-30 kDa (from AGF)				
Spot 7303	28 kDa; 5.1	gi28849953	3	VHSFFWR
Spot 7304	28 kDa; 5.0	gi28849953	9	VHSFFWR WCSLTSNYDR
Spot 8303	28 kDa; 4.9	gi28849953	15	VHSFFWR WCSLTSNYDR DEPECVPFIYR
Spot 8303	28 kDa; 4.8	gi28849953	12	VHSFFWR WCSLTSNYDR
Spot 8403	28 kDa; 4.7	gi28849953	9	VHSFFWR WCSLTSNYDR
Spermadhesin Z13 (from AGF)				
Spot 1201	14 kDa; 6.8	gi12585540	25	KPNHPAPDFPLIFR ESLEIIEGPPESSSNSR
Spot 2201	14 kDa; 6.5	gi12585540	25	KPNHPAPDFFLIFR ESLEIIEGPPESSNSR
Spot 2202	14 kDa; 6.3	gi12585540	33	DSTDGLLVK KPNHPAPDFFLIFR ESLEIIEGPPESSNSR
Spermadhesin Z13 (from seminal plasma)				
Spot 1	14 kDa; 7.5	gi12585540	37	DSTDGLLVK ESLEIIEGPPESSNSR ICDTSHAEYTSCTNTMTVK
Spot 2	13 kDa; 7.4	gi12585540	30	ESLEIIEGPPESSNSR ICDTSHAEYTSCTNTMTVK
Spot 3	13 kDa; 7.2	gi12585540	30	ESLEIIEGPPESSNSR ICDTSHAEYTSCTNTMTVK
Phospholipase A2, group VII (from AGF)				
Spot 2801	58 kDa; 6.3	gi27807045	2	IPQPLFFINSER
Spot 3801	58 kDa; 6.2	gi27807045	13	IIGYLFTLK FLGTHWLVGK IQALMAAANIGQSK IPQPLFFINSER
Spot 4801	58 kDa; 6.1	gi27807045	9	YPLIIFSHGLGAFR RGEEEFPLR FQYPSNIIR FLGTHWLVGK
Spot 4802	58 kDa; 6.0	gi27807045	15	IQALMAAANIGQSK EYFLGLSK FQYPSNIIR FLGTHWLVGKIQALMAAANIGQSK

* Spot number and position according to Figure 2 (for accessory sex gland fluid proteins) and Figure 5 (for seminal plasma proteins).
† All peptides matched to proteins identified in the *Bos taurus* species in the NCBInr database.

Spermadhesin Z13 is a peptide that displays 50% and 43% homology with the acidic seminal fluid protein and seminal plasma motility inhibitor (SPMI), respectively (Tedeschi et al, 2000). The former has positive effects on bovine sperm in vitro when at average concentrations, but it can inhibit both sperm motility and mitochondrial activity when at high levels (Schoneck et al, 1996). In humans and boars, SPMI decreases sperm movement by in-

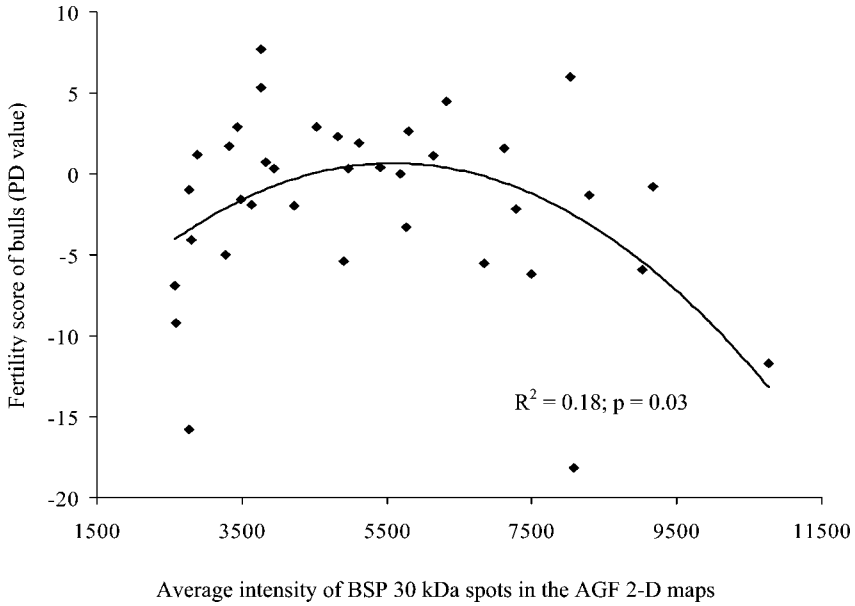

Figure 6. Relationship between the average intensity of 5 spots identified as BSP 30 kDa in 2-dimensional maps of accessory sex gland fluid and fertility scores of mature Holstein bulls.

terfering with dynein ATPase (Iwamoto et al, 1995; Robert and Gagnon, 1996). Other types of spermadhesins isolated from boar seminal plasma, such as AQN-1, AQN-3, AWN, PSP-I, and II, also influence sperm motility (Centurion et al, 2003; Caballero et al, 2004) and its oocyte-penetrating capacity (Caballero et al, 2004), suggesting the existence of a complex mechanism played by those type of proteins in the male. Regarding the reason why spermadhesin Z13 related to low fertility in the bull, clues can be obtained from the studies conducted with human and porcine peptides that share some degree of amino acid homology. However, detailed experimental data have yet to describe the functional attributes of spermadhesin Z13 expressed in the bovine accessory sex glands.

Table 2. *Empirical prediction of bull fertility based on intensity of spots in 2-dimensional protein maps of accessory sex gland fluid (AGF)*

Regression Equations That Include, as Independent Variables, Intensity of Spots* on AGF Protein Maps	R^2 and P Value
1) PD† = $-7.0 - 0.0005 \times$ (spermadhesin) $+ 0.00015 \times$ (osteopontin) $+ 0.0042 \times$ (BSP 30 kDa) $- 3.59 \times 10^{-7} \times$ (BSP 30 kDa)^2	$R^2 = .56$, $P < .0001$
2) PD = $-8.15 - 0.0005 \times$ (spermadhesin) $+ 0.0026 \times$ (phospholipase A_2) $+ 0.0048 \times$ (BSP 30 kDa) $- 4.14 \times 10^{-7} \times$ (BSP 30 kDa)^2	$R^2 = .53$, $P < .0001$

* Spot intensity given as ppm of the total integrated optical density, as calculated by PDQest software.

† PD indicates percentage point deviation from the average nonreturn rate of bulls; (spermadhesin): average intensity of two 14-kDa spermadhesins (spots 2201 and 2202); (osteopontin): intensity of a 55-kDa osteopontin (spot 9802); (BSP 30 kDa): average intensity of five BSP 30 kDa isoforms (spots 7303–8403); (phospholipase A_2): average intensity of 2 isoforms of 58-kDa phospholipase A_2 (spots 3801 and 4801), as shown in Figure 2.

The 55-kDa protein in AGF identified as osteopontin (OPN) was positively related to fertility, confirming earlier studies showing that OPN was more prevalent in the seminal plasma of higher fertility bulls (Killian et al, 1993; Cancel et al, 1997). While spermadhesin Z13 had a more linear (and inverse) distribution among groups of bulls with different fertility, OPN appeared as the most typical protein of bulls with the highest scores. In the present study, fertility data about Holstein sires were obtained when intrauterine inseminations (IUI) were performed using the usual number of sperm designed to give maximum fertility. This type of approach is obviously routine for the AI industry and makes perfect sense for most commercial uses of IUI. It is possible, however, that additional experiments designed to use only a limited number of sperm from bulls with similar characteristics, for instance 50% of the conventional dose, could reveal more detailed associations between AGF proteins and fertility. Effects of fertility and antifertility factors described here could have more pronounced influence on bulls' reproductive performance, either positive or negative, when sperm numbers are limited. Such a hypothesis needs to be tested in the future and supported by functional studies about those proteins.

Osteopontin was originally described by Senger et al (1979) in bone tissues, although its expression is also found in endothelial cells, macrophages, mammary gland,

certain tumor cells (Liaw et al, 1998; Mazalli et al, 2002; Denhardt, 2004; Wai and Kuo, 2004), follicles, corpus luteum, trophoblasts, uterine epithelium (Nomura et al, 1988; Johnson et al, 2003), and testes (Cancel et al, 1999). Evidence from these studies suggests that OPN is associated with cell adhesion, tissue remodeling, bone mineralization, immune cell stimulation and chemotaxis, cell survival, intracellular signaling, and cytoskeleton dynamics. We have recently determined (Killian, unpublished results) that incubation of bovine oocytes with oviductal follicular fluid and antibodies against OPN inhibited sperm-oocyte binding, fertilization, and embryo development, and, in a study using the OPN knockout mouse, fetal size was reduced between gestational days 10.5 and 19.5 (Weintraub et al, 2004). It seems reasonable, therefore, that OPN influences sperm-oocyte interaction and events during early embryo development and this would explain, at least partially, its association with fertility indexes of bulls.

The amount of BSP 30 kDa isoforms in AGF was linked to bull fertility, following a quadratic pattern. Bovine seminal proteins (BSPs) are known to bind to phospholipids on sperm shortly after emission, stimulating cholesterol release from sperm membrane (Thérien et al, 1998; Visconti and Kopf, 1998). In the female reproductive tract, BSP-bound sperm interacts with oviductal components and stimulates a second cholesterol efflux, resulting in capacitation (Benoff et al, 1993; Thérien et al, 1998). Thus, a positive effect of BSP 30 kDa on fertility could be linked to its ability to mediate these events, which are crucial for successful fertilization. However, based on our model, while lower amounts of BSP 30 kDa may facilitate fertilization, higher amounts of BSP 30 kDa became detrimental to fertility. This empirical conclusion is in agreement with studies showing that exposure of sperm to increasing concentrations of BSP causes damage to the sperm membrane resulting from an excess of lipid influx (Manjunath et al, 2002). Although low-density lipoproteins present in extenders, such as egg yolk and milk, protect stored sperm against the detrimental effects of BSP (Manjunath et al, 2002; Bergeron et al, 2004), we suggest that this protective action is probably not sufficient in the case of bulls with high concentrations of BSP in the accessory sex gland fluid and, consequently, in the seminal plasma. Moreover, interactions among proteins, not detected by our statistical models, may occur in the milieu of the accessory gland fluid. BSP proteins, including the BSP 30 kDa, have been shown to inhibit the activity of sperm PLA_2 in a concentration-dependent manner (Manjunath et al, 1994). We found that a secreted form of PLA_2 present in the AGF was more prevalent in bulls of high fertility. These mechanisms may explain why a quadratic effect was observed with the amount of BSP 30 kDa detected in the AGF gels.

The isoforms of PLA_2 identified in the AGF were similar to the secreted form of PLA_2 that have been purified from bovine seminal plasma (60-kDa; pI 5.6 ± 0.07; Soubeyrand et al, 1997). Another low–molecular-weight form of PLA_2 exists in membrane extracts of the bull sperm (Ronkko et al, 1991). The PLA_2 anchored to sperm membranes synthesizes arachidonic acid, which is converted to prostaglandin E_2, leading to events related to acrosome reaction (Breitbart and Spungin, 1997). Sperm PLA_2 is also implicated in sperm-egg fusion (Riffo and Párraga, 1997; Yuan et al, 2003) and secreted PLA_2 stimulates cytokine release by immune cells (Granata et al, 2005) and exerts a potent antimicrobial action in the seminal plasma (Weinrauch et al, 1996; Bourgeon et al, 2004). In general support of an association between PLA_2 and fertility, a recent study reports that the $PLA_2\beta$-gene knockout mouse has sperm with impaired motility and reduced capacity to fertilize oocytes both in vitro and in vivo (Bao et al, 2004).

In summary, we determined that specific proteins expressed in the accessory sex gland fluid accounted for a significant proportion of the variation in fertility indexes of dairy sires. We confirmed earlier findings that osteopontin is related to bull fertility and identified spermadhesin, BSP 30 kDa, and phospholipase A_2 as new markers of male fertility. Although known functional attributes of these proteins provide some understanding of how they may influence male reproductive performance, it was beyond the scope of the present study to test experimentally how these proteins affect sperm function and fertilization. These objectives will be pursued in future investigations.

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