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Seminal Plasma Proteins as Potential Markers of Relative Fertility in Boars[1]

Running Title: Boar Fertility Markers in Seminal Plasma

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ABSTRACT

This study investigated whether specific proteins from distinct seminal plasma fractions of boars could be related to in vivo fertility. Nine boars with acceptable sperm motility and morphology for use in AI demonstrated major differences in total born and pregnancy rate when low-sperm doses (1.5 billion sperm) were used to breed a minimum of 50 gilts per boar. The two lowest and two highest fertility boars were chosen for evaluation of specific seminal plasma proteins. On four occasions, semen was collected and separated into three fractions based on sperm concentration (Sperm-peak, Sperm-rich and Sperm-free), and the fractions were analyzed for total protein concentration, and abundance of Major Seminal Plasma Glycoprotein (PSP-I), AWN-1 and Osteopontin protein using Western blotting techniques. The concentrations of these seminal plasma proteins was lower in the Sperm-Peak fractions compared to the Sperm-Free fractions ($P < 0.05$). Seminal plasma from the pooled sperm-rich fraction used for artificial insemination was also subjected to two-dimensional gel electrophoresis to investigate novel protein markers related to in vivo fertility. Total piglets born ($r = -0.76$, $P = 0.01$) and sperm motility at day 7 ($r = -0.74$, $P = 0.037$) were again negatively correlated with a 22 kDa protein identified by mass spectrometry as PSP-I. However, Fertility Index and farrowing rate tended to be positively correlated ($P < 0.10$) with a 25 kDa protein, identified as Glutathione Perioxidase (GPX-5), an antioxidant enzyme which may protect sperm membranes from oxidative damage. These candidate proteins merit further investigation as markers of fertility in boars.

Key Words: *male fertility, artificial insemination, proteins, semen, pig, 2-D gel electrophoresis, tandem mass spectrometry*

Introduction

Progress has been made in developing reliable indicators of ejaculate quality that allow exclusion of low quality ejaculates for use in AI. Physical semen characteristics and sperm morphology measurements are not always indicative of fertility and reproductive performance in boars, and accurate and predictive genetic and protein markers are still needed (as reviewed by Foxcroft et al., 2008). Specific proteins in seminal plasma have been identified as potential markers of male fertility or infertility in the human (Martínez-Heredia et al., 2008; Yamakawa et

al., 2007). Comprehensive proteomic analyses have been conducted in the bull (Moura et al., 2007) and stallion (Fouchécourt et al., 2000) to identify proteins in the male accessory gland fluids. In the pig, initial characterization of proteins in boar seminal plasma has begun (Strzezek et al., 2005); however to date, no seminal plasma proteins identified in boar seminal plasma have been associated with fertility in vivo.

Seminal plasma is composed of secretions from the male accessory sex glands and epididymis, which contains many organic and inorganic components that have effects on sperm quality (Foxcroft et al., 2008). The proteins secreted into seminal plasma may play an important role during sperm capacitation and fertilization (Rodríguez-Martínez et al., 1998), and may also serve to protect sperm from damage or to maintain their longevity. In the boar, the ejaculate consists of a large volume of semen with a relatively dilute sperm concentration. Boar semen is ejaculated in specific sequential fractions namely the first sperm-rich fraction, followed by the relatively sperm-free fraction prior to the second sperm-rich fraction, and then the gel fraction at the end of the ejaculate collection. Pig semen destined for use in artificial insemination is often comprised of the pooled first sperm-rich fraction, while the remainder of the ejaculate is usually discarded. During natural mating, the sperm are usually only exposed to proteins in the initial sperm-rich fraction, and it has been shown the exposure of sperm to the later fractions of the ejaculate have a detrimental effect on sperm quality and relative fertility (as reviewed by Rodríguez-Martínez et al., 2008). For example, previous studies from our laboratory showed differences in oocyte penetration rates *in vitro* when sperm were pre-incubated with different fractions of seminal plasma from the same ejaculate (Zhu et al., 2000). One of the goals of this study was to evaluate the specific protein content of these different fractions to better understand their individual contributions to fertility potential and the roles of these proteins in fertility.

Using 2D gel electrophoresis, Killian et al. (1993) identified two seminal plasma proteins with high fertility in bulls (26 kDa and 55 kDa) and two proteins that were correlated with low fertility (16 kDa and 16 kDa). The 55kDa fertility-associated protein has been identified as osteopontin (Cancel et al., 1999) and the 26kDa fertility-associated protein as Lipocalin-Type Prostaglandin D synthase (Gerena et al., 1998). In stallions, Brandon et al. (1999) reported that a 72 kDa seminal plasma protein, also identified as horse osteopontin was positively correlated with fertility. Osteopontin has recently been localized on ejaculated bull sperm and may play a role in fertilization and also as a block to polyspermy (Erikson et al., 2007). In the pig, addition

of osteopontin during in vitro fertilization also reduced polyspermy rates (Hao et al., 2006) and improved embryo development after fertilization (Hao et al., 2008).

Specific proteins from the spermadhesin family, such as porcine seminal protein (PSP), AWN, and AQN, coat the sperm surface during ejaculation, producing structural changes to the sperm plasma membrane that affect sperm performance during the fertilization process (Manásková et al., 2003). The Major Seminal Plasma Glycoprotein (PSP-I), isolated and identified from boar seminal plasma by Rutherfurd et al. (1992), may prevent premature capacitation and the acrosome reaction (Kwok et al., 1993; Töpfer-Petersen et al., 1998). It may also have immuno-regulatory activity (Kwok et al., 1993; Yang et al., 1998; Assreuy et al., 2002, 2003). AWN-1 is another spermadhesin that affects zona-pellucida-binding activity (Sanz et al., 1992; Rodríguez-Martínez et al., 1998), and may also have a role in the capacitation process (Töpfer-Petersen et al., 1998; Calvete et al., 1997). AWN-1 is synthesized by the rete testis, prostate, seminal vesicles (Sinowatz et al., 1995) and female reproductive tract (Ekhlasi-Hundrieser et al., 2002). Given the functions of these spermadhesins, they may potentially be candidate markers of boar fertility.

The main objective of the present study was to evaluate specific seminal plasma proteins, PSP-I, AWN-I and Osteopontin, in different ejaculate fractions that could be effective predictors of relative boar fertility using a population of boars that; 1) would be considered acceptable for use in AI programs on the basis of ejaculate/sperm characteristics measured in most commercial AI centers, and 2) have established differences in *in vivo* fertility when only 1.5 billion sperm per AI dose are used for insemination (Ruiz-Sánchez et al., 2006). The seminal plasma proteome in the pooled sperm-rich fraction used for artificial insemination was also compared across boars of low and high fertility to further investigate potential markers of fertility.

Materials and Methods

Boar Fertility Evaluation In Vivo

The boars used in the present study for analysis of seminal plasma proteins were from the study of Ruiz-Sánchez et al. (2006). The experiment was conducted in accordance with the Canadian Council on Animal Care guidelines and with the approval of the university Animal Care and Use Committee: Livestock. Briefly, nine Genex Large White boars were housed at the

Swine Research and Technology Center (University of Alberta, Canada) and were evaluated in three groups of three boars each, during a 6.5 ± 0.1 mo period from May to February. A standardized AI protocol was used to collect semen twice-weekly from these nine boars and breed 50 ± 5 gilts per boar to determine in vivo fertility differences among boars. The boars were assessed on several in vivo fertility parameters including the average total born, total born alive, pregnany rate as determined by ultrasound at day 30 of pregnancy, farrowing rate, and a fertility index (the total piglets born as a proportion of the number of gilts initially bred by each boar).

Semen Collection for Seminal Plasma Evaluation

Ejaculates were collected into a series of sterile pre-warmed 15-mL Falcon tubes (VWR Canlab, Mississauga, Ontario) as described by Xu et al. (1996; 1998; Figure 1) and three fractions were defined on the basis of sperm concentration (Ruiz-Sánchez et al., 2006). Briefly, tubes containing the first Sperm-Rich fraction were identified both visually and using a calibrated colorimeter (model 254 Sherwood Scientific Ltd. Cambridge, UK). The Sperm-Peak fraction was considered to be the tube containing the highest sperm concentration within the Sperm-Rich fraction (usually the first 10 ml fraction collected). The last tubes of the first Sperm-Rich fraction were measured to identify the final tube with a concentration of $\geq 100 \times 10^6$ spermatozoa per mL; this and the previous tubes were included as part of the first Sperm-Rich fraction. Finally, tubes containing less than 100×10^6 spermatozoa per mL, between the first Sperm-Rich fraction and the second Sperm-Rich fraction, were considered to be part of the Sperm-Free fraction. All the tubes from the first Sperm-Rich fraction were then combined in a pre-warmed thermos, filtered through gauze to eliminate any gel component, and this combined first Sperm-Rich fraction was then used for routine semen evaluation (twice a week during the evaluation period), and for breeding by AI (every three weeks out of four).

On four occasions during the evaluation period, 3 mL aliquots of each identified fraction (Sperm-Peak; the combined Sperm-Rich, and the Sperm-Free) were used for seminal plasma protein evaluation. After collection, semen samples were centrifuged at 1,020 x g for 30 min at 4°C and the seminal plasma supernatant recovered was frozen with liquid nitrogen and stored at –20°C until analysis.

Seminal Plasma Protein Evaluation In Vitro

Seminal plasma samples were thawed and immediately centrifuged at 10,000 x g for 15 min to remove any cellular debris. Total protein concentration was then quantified using the Pierce BCA Protein Assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer's directions.

PSP-I Analysis by Western Blot

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

The PSP-I antibody was kindly donated by Dr Kwok (Department of Obstetrics and Gynecology, Albert Einstein Medical Center, Philadelphia, USA). PSP-I was evaluated in both glycosylated and deglycosylated samples from the three fractions and four time periods (collections) from each of the nine boars. Both deglycosylated and glycosylated samples were separated on 15% SDS-polyacrylamide gels. A total of 5 μg of total protein from each sample and a control (pooled) sample were loaded onto each gel. After electrophoresis, proteins were transferred to Hybond ECL nitrocellulose membrane (GE Healthcare, Quebec, Canada) using a constant current of 75mA for 15 h at 4°C. Membranes were blocked with Tris buffered saline (TBS) supplemented with 0.1% (v/v) Tween 20 (TBS-T) and 5% (w/v) non-fat milk for 1 h at room temperature. Membranes were then washed three times (5 min each) with TBS-T and incubated for 1 h with primary antiserum raised against PSP-I (1:20,000 dilution), followed by three 5-min washes. The membranes were then incubated with anti-rabbit IgG, peroxidase-linked donkey antibody (GE Healthcare) for 30 min at room temperature (1:16,000 dilution), and

washed three times with TBS-T for 5 min. Immunoactivity was detected by ECL chemiluminescence (GE Healthcare) according to the manufacturer's instructions. The membrane was exposed to Hyperfilm-ECL (GE Healthcare) for 10 sec and developed. The films were scanned using an Imaging Densitometer (Bio-Rad Labs, Hercules, CA) and immunoreactive PSP-I bands were quantified using densitometry analysis software (Molecular Analyst v2.01, Bio-Rad Labs, Hercules, CA). To standardize the gels, an internal control (pooled) sample was run in each blot. The relative abundance of PSP-I (arbitrary units per μg of total protein) was calculated using the volume (optical density by surface area) of the sample PSP-1 band divided by the volume of the internal control band, divided by the amount of protein loaded (5 μg). The PSP-I concentration per mL of seminal plasma (PSP-I/mL) was estimated as PSP-I abundance x the total protein concentration. Finally, the total amount of PSP-I per AI dose (PSP-I/AI dose) was calculated as PSP-I/mL x estimate volume of seminal plasma included in each AI dose of semen. AWN-1 and osteopontin protein concentrations were calculated in the same manner.

AWN-I Analysis by Western Blot

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

Osteopontin Analysis by Western Blot

Equivalent samples from the same four boars analyzed for AWN-1 were also evaluated for osteopontin (OPN), again using the PSP-I Western blot protocol with the following variations based on Johnson et al. (1999). Proteins (25 μg) were loaded onto each well and separated on a

15% polyacrylamide gel. A sample of sow's milk was also used as an OPN positive control. As osteopontin is known to be enriched in milk. The membranes were initially blocked overnight at 4°C with TBS-T 5% non-fat milk, incubated overnight with a cocktail of rabbit polyclonal antibodies against recombinant human OPN (LF-123 and LF-124; 1:1,000; Fisher et al., 1995) and then incubated for 2 h at room temperature with a peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:10,000). The membrane was exposed to a Hyperfilm ECL for 15 sec. Specificity of the 1st antibody was previously tested by Cancel et al. (1997) to identify OPN in bull semen and by Johnson et al. (1999) to identify OPN in the uterus.

2-D Gel Electrophoresis of Seminal Plasma Proteins

Proteomics analysis was restricted to the Sperm-Rich fraction of the same samples used for AWN-1 and OPN analysis as this was representative of the seminal plasma proteins in the dose used for artificial insemination. The sperm rich fraction was extracted using the same acetone precipitation method described earlier and 100 µg of protein was solubilized in rehydration buffer (7 M urea, 2 M thiourea, 4 % (w/v) chaps, 0.5 % (v/v) pharmalytes pH 3-10, and 20 mM DTT for 1 h at room temperature, before loading the sample onto 7 cm linear pH 3-10 Immobiline (GE Healthcare) strips for overnight rehydration. The first dimension separation program on an Ettan IPGPhor isoelectric focusing apparatus (GE Healthcare) f was 10 min at 500 V, 10 min at 1,000 V, 1.5 h at 4000 V and 1 h at 5,000 V for a total of 11, 250 Vhr. After focusing, strips were equilibrated in 50 mM Tris-HCl, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and bromophenol blue, for 15 min with 1% (w/v) DTT, and then alkylated using 2.5 % (w/v) Iodoacetamide for 15 min. The gel strips were then loaded onto stimulaneously cast 12% SDS-PAGE gels to separate proteins in the second dimension according to their molecular weight. The 7 cm strips were all run simultaneously using a ETTAN DALT-six gel electrophoresis system (GE Healthcare), with three 7 cm strips run on each of six 24 cm slab gels in the second dimension. The resulting gels were then fixed overnight and proteins visualized using silver staining.

For each gel, the protein spots were quantified using Imagemaster 2-D Elite analysis software (GE Healthcare). The individual volume measurements (in relative units) for each protein were corrected for the total spot volume on each gel, and were also normalized across

gels using same reference gel. The spot volume measurements were exported into SAS for analysis.

LC-MS/MS Identification of Seminal Plasma Proteins

A preparative 2D gel was run with 1mg of total protein on a 24 cm Immobiline DryStrip Gel (GE Healthcare), in the linear pH 3-10 range using an extended electrofocussing protocol for 65,000 Vhrs, and loaded onto a 12 % SDS-PAGE slab gel. The resulting gel was fixed overnight in 50 % (v/v) methanol and stained using Bio-Safe Coomassie Blue (Bio-Rad Labs). The protein spots of interest were manually exised and sent to a mass spectrometry facility for further processing and identification (Centre Genomique du Quebec, Sainte-Foy, Canada). Tryptic digestions of the proteins were performed on a MassPrep liquid handling robot (Waters, Mississagua, Canada) according to the manufacturer's specifications and using sequencing grade modified trypsin (Promega, Madison, WI).

Peptide extracts were separated by online reversed-phase nanoscale capillary LC and analyzed by electrospray MS (ES MS/MS) using a LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA) equipped with a nanoelectrospray ion source (Thermo Electron, San Jose, CA, USA). Peptide separation took place within a PicoFrit column BioBasic C18, 10 cm x 0.075 mm internal diameter (New Objective, Woburn, MA, USA) with a linear gradient from 2 % to 50 % solvent B (acetonitrile, 0.1 % formic acid) in 30 min, at 200 nl/min. Mass spectra were acquired using data-dependent acquisition mode (Xcalibre software, version 2.0). Each full-scan mass spectrum (400–2000 m/z) was followed by collision-induced dissociation of the seven most intense ions. The dynamic exclusion function was enabled (30 s exclusion), and the relative collisional fragmentation energy was set to 35 %.

Interpretation of Tandem MS Spectra

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.0). The database used to search tryptic peptides was the mammalian protein database Uniref_100_14_Mammalia_40674. Fragment and parent ion monoisotopic mass tolerance were, respectively, of 0.5 Da and 2.0 Da. Iodoacetamide derivative of cysteine was specified as a fixed modification and methionine oxidation were specified as variable modifications. Two missed cleavages were allowed.

Criteria for protein identification

Scaffold (version Scaffold_2.1.03, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0 % probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0 % probability and contained at least 2 unique identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Statistical Analysis

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

The Insight procedure of SAS was used to evaluate the correlation between fertility in vivo (pregnancy rate, farrowing rate, total pigs born, and Fertility Index) and seminal plasma proteins (total protein content, PSP-I, AWN-I, osteopontin and protein abundance in the 2D gels).

Results

Fertility Evaluation In Vivo

For ease of interpretation of the *in vivo* fertility performance of the boars used in this study, Table 1 is a summary of the results presented in Ruiz-Sánchez et al. (2006). Based on the Fertility Index of the nine boars, the two lowest (R-1, G-1) and two highest fertility (R-2, Y-2) boars were chosen for analysis of specific proteins (AWN-1, osteopontin and 2D gels).

Seminal plasma analysis

Different semen fractions (sperm-peak, sperm-rich and sperm-free) have been shown to result in differential fertility and sperm quality when sperm are pre-incubated with these fractions independently. As a result, the specific proteins, PSP-I, AWN-I, and osteopontin, were analysed within these discrete fractions to determine if there were differences between fractions that related to boar fertility in vivo. The seminal plasma proteome was restricted to analysis within the sperm-rich fraction only, as this was the fraction that was used for artificial insemination.

Western Blotting Analysis

The antiserum raised against AWN-1 showed immunoactivity against a specific band at 14 kDa (Figure 3). Finally, the use of the polyclonal antiserum raised against the N-terminus (LF-124) and C-terminus (L-123) of human recombinant OPN showed immunoactivity against three protein bands of 70, 12 and 9 kDa (OPN-70, OPN-12 and OPN-9, respectively) (Figure 4).

Seminal Plasma Evaluation: Total Protein and D-PSP-I

Total protein concentration and PSP-I abundance were analysed across all nine boars in the study within seminal plasma fractions. Theis associations with relative fertility were also established. For Western blotting, the specific antiserum against PSP-I showed immunoactivity against both glycosylated (PSP-I) and deglycosylated PSP-I proteins (D-PSP-I) in the range of 14 to 20 kDa and 12 kDa respectively (Figure 2). There were no statistical differences between PSP-I and D-PSP-I, and thus D-PSP-I values were chosen to represent the abundance for that protein throughout the paper.

Concentration of total protein and D-PSP-1 were lower ($P < 0.05$) in the Sperm-Peak,

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

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

The *in vitro* fertilization (IVF) data collected and presented in Ruiz-Sánchez et al. (2006) were also compared with the data from seminal plasma proteins for all nine boars. This comparison established that total protein concentration was negatively correlated with zona pellucida penetration rate and average number of sperm penetrating the zona (r = -0.47 and r = -0.50, respectively; P < 0.05). There was also a negative relationship between D-PSP-I abundance and average number of sperm penetrating the zona (r = -0.38, P < 0.05).

Seminal Plasma Evaluation: AWN-1 and Osteopontin

For Western blotting, the antiserum raised against AWN-1 showed immunoactivity against a specific band at 14 kDa (Figure 3). The use of the polyclonal antiserum raised against the N-terminus (LF-124) and C-terminus (L-123) of human recombinant OPN showed immunoactivity against three protein bands of 70, 12 and 9 kDa (OPN-70, OPN-12 and OPN-9, respectively) (Figure 4).

Comparisons of seminal plasma fractions demonstrated that OPN-9 and OPN-12 concentrations were lower (P < 0.05) in the Sperm-Peak fraction than in the other two fractions

(Table 2). Concentrations of AWN-1 and OPN-70 were lower ($P < 0.05$) in the Sperm-Peak fraction than the Sperm-Free fraction, whilst concentrations in the Sperm-Rich fraction were intermediate and not different to the other two fractions (Table 2). No difference in any measure of protein content among the highest and the lowest fertility boars in the Sperm-Free and Sperm-Peak fractions were established (Table 3), whereas differences were found for the Sperm-Rich fraction in AWN-1 concentration, and the lowest fertility boars had the highest amount of AWN-1, OPN-9 and OPN-12 per AI dose.

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

2-D Gel Electrophoresis of Seminal Plasma Proteins

Figure 5A is a representative gel showing two-dimensional separation of seminal plasma proteins for the second collection from Boar R-1. From a qualitative perspective, all seminal plasma protein species were identified in both the highest and the lowest fertility boars. Quantification of the 42 numbered proteins established differences ($P < 0.05$) among boars for protein 7 (46 kDa, pI 6.9), 17 (10 kda, pI 9.0), 22 (18 kDa, pI 9.2), and 24 (27 kDa, pI 7.6). One of the lowest fertility boars, R-1, had lower abundance of seminal plasma proteins 7, 17 and 22 ($P < 0.05$) than the other three boars, but there were no differences in relative abundance ($P > 0.05$) of these proteins when analyzed by fertility class.

Western blot analysis performed on a 2D gel shown in Figure 4A, localized PSP-I as a group of glycoslylated proteins ranging from 12 to 21 kDa and a basic pI range of 7 to 10, which appeared to include proteins 22 and 17 (Figure 5B). Analysis of proteins 17 and 22 by mass spectrometry confirmed the identity of both proteins as Major seminal plasma glycoprotein (PSP-1) (Table 4).

When correlations with *in vivo* characteristics were considered, protein 4 (60 kDa, pI 6.5) was negatively correlated with farrowing rate (r = -0.66, P = 0.04) and with the Fertility Index (r = -0.66, P = 0.04), while protein 27 (22 kDa, pI 6.0) had a strong negative relationship with total piglets born (r = -0.77, P = 0.010). In addition, the semen characteristics and extended storage data collected and presented in Ruiz-Sánchez et al. (2006) were also compared with the data from seminal plasma proteins from these boars, and sperm motility at day 7 of storage was also negatively correlated (r = -0.74, P = 0.037) with Protein 27. The identity of Protein 27 was confirmed to be PSP-I by mass spectrometry techniques, whereas protein 4 did not reveal a positive identification (Table 4). In contrast, protein 26 (26 kDa, pI 5.9) tended to be positively correlated with pregnancy rate (r = 0.454, P = 0.09), farrowing rate (r = 0.450, P = 0.09) and Fertility Index (r = 0.481, P = 0.07). Protein 26 was confirmed to be Glutathione Peroxidase (epididymal androgen-related protein) (GPX5), the only selenium-independent epididymis-specific glutathione peroxidase, by mass spectrometry (Table 4).

Discussion

As a part of the fertilization process seminal plasma proteins play an important role in sperm reservoir formation, sperm capacitation, and sperm-oocyte interactions (reviewed by Foxcroft et al., 2008; Rodriguez-Martinez et al., 2008). Specific seminal plasma proteins have previously been identified as potential markers of male fertility in the bull (Killian et al., 1993) and stallion (Brandon et al., 1999). The present study identified specific proteins in the sperm-rich fraction of seminal plasma, such as PSP-I and GPX5, to be associated either negatively or positively with boar fertility *in vivo*, providing the basis to use them as a complementary tool to identify sires with high and low relative fertility that could have considerable impact on reproductive efficiency. Futhermore, the abundance of three specific proteins thought to be related to fertility, AWN-1, PSP-I and OPN, were also characterized in three distinct fractions of the boar ejaculate to elucidate differences observed between these fractions on relative fertility and sperm quality in the boar.

In the context of the data reported from the present study, it is important to emphasize two key points. Firstly, as described by Ruiz-Sanchez et al. (2006) the quality of the ejaculates used in the present study (>80% progressive motility and >85% morphologically normal sperm) exceeded normal industry standards (>70 % progressive motility and <30 % abnormal sperm) for

use in AI. Secondly, boar fertility was evaluated *in vivo* using a low sperm dose (1.5 billion morphologically normal, and motile, sperm per AI dose), to avoid compensable traits that are masked if higher sperm doses are used. However, the differences observed with in vivo performance of these boars may have been more pronounced with an even further reduction of sperm dose, as shown in Tardif et al., (1999). It is essential to recognize that these specific conditions may identify a different subpopulation of relatively less fertile boars than in previous studies.

In the present study the collection of the ejaculates in discrete sequential fractions allowed further characterization of differences in seminal plasma proteins among these fractions as previously reported by our laboratory (Zhu et al., 2000). The Sperm-Peak fraction containing the highest sperm concentration is usually the first tube collected, and sperm from this fraction have the highest fertilization ability in vitro. The Sperm-Peak fraction contained lower total protein than the other two fractions and lower concentrations of all specific proteins than the Sperm-Free fraction. One explanation for these differences could be the origin of the seminal plasma components with respect to the different accessory sex glands and their sequential contribution of secreted proteins to the ejaculate. The first secretions present in the ejaculate are produced by the prostate gland which serves to clear the male urino-genital tract, followed by very high sperm concentrations in the first 10 to 15 mL of the Sperm-Rich fraction. The ejaculate then becomes increasingly diluted by seminal vesicle fluid in the next 30 to 70 mL of the ejaculate, until essentially sperm-free fluid is collected in the Sperm-Free fraction. Depending on the frequency of collection, a second, less concentrated Sperm-Rich fraction may be present, after which the ejaculate ends with a gel fraction being secreted from the bulborourethral glands. Prostate secretions contain lower concentrations of PSP-I and AWN-1 than seminal plasma of the complete ejaculate (Manásková et al., 2002). These results support our observations that the Sperm-Peak fraction, the first sperm fraction collected that contains mainly prostate secretions, had lower concentrations of PSP-I than the Sperm-Rich fraction. Similarily, Rodríguez-Martínez et al., (2008) have also reported consistently higher protein concentrations in the sperm-rich compared to the sperm-peak fractions.

A second factor that could contribute to the protein differences between fractions is the variability in the concentration of sperm. The high concentration of sperm in the Sperm-Peak fraction would retain a higher proportion of proteins onto their membranes, thus reducing the

residual amount of total protein in seminal plasma. Metz et al. (1990) reported that epididymal sperm adsorbed 14 pg protein/sperm over a 10 min period and that 82% of the proteins retained were low molecular weight proteins. These authors also confirmed that low quality sperm (sperm progressive motility of 50 to 30% after washing with Tyrode's solution) adsorbed significantly less protein (3 pg protein/sperm per 10 min). This relationship is consistent with our observations that sperm concentration and total protein concentrations were negatively related in the Sperm-Rich and Sperm-Peak fractions. Therefore, a combination of three factors: 1) seminal plasma origin, 2) sperm quality (sperm ability to absorb seminal plasma proteins), and 3) sperm concentration, collectively determine the variation in total protein concentration among the seminal plasma fractions.

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

Osteopontin (OPN) has been detected in both female (Johnson et al., 1999; Garlow et al 2002) and male (Cancel et al., 1999; Rodríguez et al., 2000; Luedtke et al., 2002) reproductive tracts. OPN has been identified as a seminal plasma protein that is positively associated with fertility in the bull (55 kDA, pI 4.5) (Cancel et al., 1997; Cancel et al., 1999) and the stallion (SP-1, 72 kDA, pI 5.6) (Brandon et al., 1999). In the present experiment, western blot analysis with an antiserum against human OPN, identified three immunoactive bands at 70, 12 and 9 kDa. A 70 kDA form of OPN has been reported in uterine flushings in the pig (Garlow et al., 2002), and appears to be homologous to SP-1, a stallion seminal plasma osteopontin (Brandon et al., 1999). Although the 70 kDA osteopontin species identified in the present study of boar seminal plasma is homologous to the bull (55 kDA) and stallion (70 kDA) OPN form, the relative

amount of OPN-70 did not differ among boars. Similarily, under the conditions used, none of the higher molecular weight proteins, or those in the area of osteopontin (70 kDa, pI 4-6) in the seminal plasma proteome as analysed by 2D gels were associated with differences in fertility. The lack of an association between osteopontin abundance and *in vivo* fertility in our study is likely due to the use of boars with a narrow range of fertility, thus excluding the more infertile boars which would likely reveal a higher variability in seminal plasma protein abundance. All of the ejaculates used in the present study exceeded normal industry standards for use in AI and the differences in fertility *in vivo* were established using low numbers of sperm per AI dose. As there were no major differences in the relative abundance of AWN-1, OPN-12 and OPN-9 in the ejaculates collected, differences in the concentration of these specific proteins in seminal plasma from the highest and lowest fertility boars, may largely reflect differences in total protein concentration, and the sperm concentration of each ejaculate, which ultimately determines the amount of seminal plasma present after dilution of the ejaculate to 1.5 billion sperm per 50mL AI dose.

The analysis of seminal plasma proteins using proteomics techniques provides important preliminary data on the abundance of specific proteins in seminal plasma, without having to rely on existing antibodies for detection (Fouchécourt et al., 2000; Moura et al., 2007; Martínez-Heredia et al., 2008). This approach has also been successfully used to identify proteins that were associated with fertility in the stallion (Brandon et al., 1999), and the bull (Killian et al., 1993). In this study, analysis of 42 different proteins in the seminal plasma of the two lowest and two highest fertility boars, demonstrated that variations in the seminal plasma proteome showed clear associations with differences in boar fertility. Although four proteins were initially identified as differing among boars, none of these proteins were specifically related to fertility. Two of the proteins, protein 17 and protein 22 were located in the same molecular size range and pI as PSP-I, and preliminary immunoblots confirmed the presence of PSP-I in this area. Further analysis by mass spectrometry confirmed these proteins as PSP-I. The significant differences observed in PSP-I between boars, lacked associations with overall fertility. This suggests that the abundance of PSP-I appears to be related to the total protein concentration in the seminal plasma of these boars. Lower fertility boar R-1, consistently had total and specific protein concentrations similar to the two higher fertility boars, whilst the other lower fertility boar G-1 exhibited higher total and specific protein concentrations compared to all three boars.

Interestingly, protein 27 (20 kDa, pI 6.0), identified by 2-D gel analysis in the present study, showed a strong negative correlation ($r = -0.7627$, $P = 0.01$) to total litter size born, but not to other fertility traits included in the overall Fertility Index. Protein 27 was also identified as PSP-I, which supports our hypothesis that PSP-I may limit fertility due to an inhibitory effect of these spermadhesins on sperm performance in a low sperm insemination dose scenario. Specific seminal plasma components have been described as decapacitation factors in humans (Zhu et al., 2006), mice (Huang et al., 2007), and other species (as reviewed in Töpfer-Petersen et al, 1998) and are assumed to protect sperm from factors in the female tract that could trigger early capacitation, thus reducing the possibility of sperm-oocyte binding. These suggestions are consistent with the observations that pre-incubation of sperm from the Sperm-Peak fraction with seminal plasma from the Sperm-Free fraction reduced oocyte penetration rate *in vitro* (Zhu et al., 2000), whereas inclusion of seminal plasma to sperm samples sorted by flow-cytometry increased the percentage of uncapacitated, acrosome-intact sperm and reduced oocyte penetration rate *in vitro* (Maxwell and Johnson, 1999). In the present experiment, total protein concentration of seminal plasma was negatively correlated with both zona pellucida penetration rate and number of sperm penetrating the zona, suggesting that an increase in total protein content produces a predominantly decapacitation effect. As a consequence, the increase in uncapacitated sperm could decrease fertilization rate *in vitro*. No differences were reported in sperm viability and mitochondrial activity when PSP-I was incubated with a highly diluted sperm sample (García et al., 2003). However, our unpublished observations have shown a negative correlation between the number of sperm attaching to each oocyte, and PSP-I in seminal plasma, which is consistent with the observation in the present study that D-PSP-I abundance was negatively correlated with both *in vivo* and *in vitro* fertility. Direct evaluation of sperm capacitation state, and the populations of specific seminal plasma proteins on the sperm membrane before and after capacitation, is needed to better understand the specific functions of seminal plasma proteins during the fertilization process.

During natural mating and insemination, since the sperm peak fraction is the first part of the ejaculate and arguably the main source of the sperm that colonize the sperm reservoir in the oviduct, it is logical to assume that these sperm are not exposed to the seminal plasma that follows in the remainder of the ejaculate (Rodriguez-Martinez et al., 2008). The “sperm-peak” and “P1” fractions exhibit positive effects on fertilization and both have lower protein

concentrations and PSP-I abundance, which suggests that these higher concentrations of certain proteins, especially the spermadhesins, have negative effects in an in vivo setting. The proteins may play an important role in sperm longetivity as depacitation factors; however, these proteins may also reduce fertility if in excess or if sperm are exposed to them for an extended period of time. This negative relationship between lower total protein and specific PSP-I, AWN-I and OPN concentrations in the sperm-peak fractions and higher relative fertility needs to be further evaluated.

Consistent with the observed negative relationships between seminal plasma proteins and fertility, the relative abundance of protein 4 (60 kDa, pI 6.5) exhibited a strong negative correlation with both farrowing rate and the overall Fertility Index. The mass spectrometry analysis failed to identify this protein, due to low protein abundance and contamination with keratin. The identity of this protein is still under investigation.

In contrast, Protein 26 (25 kDa, pI 5.9), as identified by proteomics techniques in the present study, tended to be positively associated with farrowing rate and the overall Fertility Index. Although we have previously speculated that this protein may be lipocalin-type prostaglandin D synthase (Foxcroft et al., 2008), this study confirmed its identity using mass spectrometry as Glutathione Peroxidase-5. Glutathione Peroxidase-5 is a selenium-independent free radical scavenger that is restricted to the male reproductive tract (Grignard et al., 2005). It has been shown to bind to the head of the spermatozoa in mice (Vernet et al., 1997) and is thought to protect the sperm plasma membrance from attacks by free radicals (Grignard et al., 2005). Other studies have detected another glutathione peroxidase, phospholipid hydroperoxidase (GPX4) in seminal plasma of boars and are investigating its role in sperm quality (Bailey et al., 2005; Dube et al., 2004). Boar sperm are particularily sensitive to oxidative damage (Strzezek et al., 2005), which is of interest in situations when semen is to be extended for use in AI or for cryopreservation. The inclusion of seminal plasma during cryopreservation may serve to protect the sperm membranes and the same concept may apply to the protection of sperm membranes by seminal plasma in extended semen. In Ruiz-Sánchez et al. (2006), we demonstrated that the higher fertility boars exhibit higher sperm motility in semen extended to day 7 and day 10 compared to the lower fertility boars, suggesting that the this enzyme could play a role in the protection of sperm membranes and keep sperm motile in extended semen for longer periods of time.

The overall results presented in this study and the *in vitro* fertility performance of these boars in Ruiz-Sánchez et al. (2006) provide strong evidence of substantial differences in boar fertility that cannot be identified by existing laboratory techniques used in commercial boar studs. However, inadvertent use of the relatively sub-fertile boars identified in this study would substantially affect breeding herd performance. The proteomic analysis reported in the present paper provides some of the first evidence of specific boar seminal plasma proteins that may mediate observed differences in semen quality. The combined results from this study confirm that there is no single test that can apparently predict sperm quality or boar fertility among groups of relatively fertile boars. Fertilization in mammals is a complex process, involving multiple interactions between the sperm and the seminal plasma components of the ejaculate. Furthermore, extensive interactions between components of the ejaculate and the female reproductive tract *in vivo*, are very different to the conditions in which sperm maturation is carried out *in vitro*. Therefore, semen characteristics that favour successful IVF, may not necessarily favour optimal fertility when the same sperm are used for AI.

For artificial insemination purposes, either the first sperm-rich fraction or the whole ejaculate may be collected; however in a natural mating scenario, the sperm would typically only become in contact with the sperm-rich fraction of the ejaculate (Foxcroft et al., 2008). Thus, it may become important when collecting semen to collect only the first sperm-rich fraction rather than the whole ejaculate, especially if the second fraction of the ejaculate may contain components that are inhibitory to overall boar fertility. Also, from a practical perspective, if the trend towards using lower sperm numbers for intra-uterine insemination continues, it will be essential to identify boars that are deemed less fertile in this situation, if the benefit of using fewer, but higher indexed, boars is to be realized. Information on the balance of proteins that determine the rate of capacitation and those that ultimately allow effective sperm binding to the zona pellucida and to the oocyte, will undoubtedly help to improve the selection of such high impact boars. The results presented in this paper identify several seminal plasma proteins that merit further investigation as markers of ejaculate quality and boar fertility.

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Figure Legends

Figure 1. Diagram of the three fractions of semen that were collected. The first sperm rich fraction was identified as the first series of 15 mL tubes collected from the ejaculate that contained more than 1 X 10^6 sperm. The sperm peak fraction was identified as the tube containining the highest sperm concentration within the first sperm rich fraction. The sperm-free fraction was the part of the ejaculate following the first sperm rich fraction and before the second sperm rich fraction, which contained less than 1 X 10^6 sperm per tube. The second sperm rich fraction was not used for analysis or AI. The first sperm rich fraction including the sperm peak tube were combined and used for artificial insemination.

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

Figure 3. Representative immunoblot of a 1-D SDS-PAGE gel using a specific AWN-1 antiserum and 5 mg per lane of glycosylated seminal plasma protein. Samples were from the 3rd collection from the two highest (R-2, Y-2) and the two lowest (G-1, R-1) fertility boars and from the Sperm-Peak (SP), Sperm-Rich (SR) and Sperm-Free (SF) fractions. An internal control seminal plasma pool sample (C) was included to allow comparisons across gels analyzed.

Figure 4. A representative immunoblot of one dimensional SDS-PAGE gel using a specific osteopontin antiserum, and 25 mg per lane seminal plasma protein samples from the two highest (R-2, Y-2) and the two lowest (G-1, R-1) fertility boars from the Sperm-Free fraction. Sow milk osteopontin positive control (lane 6) and an internal control seminal plasma pool sample (lane 5).

Figure 5. A: A representative 12 % 2-D SDS-PAGE gel showing seminal plasma proteins identified from the peak fraction of boar R-1. The 42 spots quantified across gels are numbered 1 to 42, B: Western blot using PSP-1 antibody on a blot transferred from a duplicate gel to that shown in Figure 4A.

Table 1. Main effects of total seminal plasma proteins and deglycosylated seminal plasma PSP-I (D-PSP-I) from the Sperm-Rich fraction of ejaculates collected during the evaluation period on relative boar fertility *in vivo*. The differences in Fertility Index for each boar are presented in rank order for comparison. Y-2 and R-2 were chosen at the two Higher Fertility Boars and R-1 and G-1 were chosen as the Lower Fertility Boars for the current study.

	Fertility *in vivo*				**Seminal plasma proteins in the Sperm-Rich Fraction**			
Boars	Pregnancy rate (%)	Farrowing rate (%)	Total piglets Born (mean ± SE)	Fertility Index (mean ± SE)	Total protein concentration (mg/mL)	Total protein per AI dose (mg/AI dose)	D-PSP-I concentration (re/mL)	D- PSP-I per AI dose (re/AI dose)
R-2	98 ^{a}	98 ^{a}	11.7 ^{ab}	11.4 ^{a}	20.0 ^{c}	72	3529 ^{a}	13276
Y-2	91 ^{ab}	89 ^{ab}	12.0 ^{a}	10.9 ^{ab}	19.4 ^{bc}	61	3432 ^{a}	10916
Pu-3	95 ^{ab}	91 ^{ab}	11.2 ^{abc}	10.2 ^{ab}	31.8 ^{ab}	135	5643 ^{b}	25510
B-1	98 ^{a}	94 ^{ab}	10.7 ^{abcd}	10.2 ^{ab}	36.7 ^{a}	126	6613 ^{b}	23152
R-3	94 ^{ab}	95 ^{ab}	10.9 ^{abcd}	10.1 ^{ab}	35.9 ^{a}	139	6025 ^{b}	23260
G-2	93 ^{ab}	91 ^{ab}	10.1 ^{abcd}	9.5 ^{ab}	36.4 ^{a}	144	6402 ^{b}	25188
B-3	93 ^{ab}	93 ^{ab}	9.6 ^{cd}	8.8 ^{abc}	31.2 ^{ab}	110	5545 ^{b}	20505
R-1	86 ^{bc}	84 ^{bc}	10.0 ^{bcd}	8.4 ^{bc}	19.0 ^{bc}	81	3434 ^{a}	15442
G-1	72 ^{c}	71 ^{c}	8.4 ^{d}	6.0 ^{c}	30.9 ^{abc}	107	5719 ^{b}	19315
± SE	-	-	0.5	0.5	6.7	35	1067	4191

Values in the table are least square means (LSM).
^{ab}: LSM with different superscripts within each column were different ($P<0.05$).
SE: pooled standard errors of LSM. re; relative expression.

Table 2. Differences in seminal plasma proteins by fraction for the selected boars, two high fertility and two lower fertility boars. Data for all nine boars in parentheses for total protein concentrations and D-PSP-1 concentrations. Seminal plasma samples were collected on four occasions per boar during the 6.5 ±1 mo evaluation period.

	Seminal Plasma Fractions for four selected boars (data for all 9 boars)			
Seminal Plasma Proteins	**Sperm-Free**	**Sperm-Rich**	**Sperm-Peak**	**± SE**
Total Protein (mg/mL)	27^{a} (33^{x})	22^{a} (28^{x})	14^{b} (16^{y})	2.8 (3.5)
D-PSP-I (re/mL)	5300^{a} (6354^{x})	3795^{ab} (5069^{x})	2530^{b} (2887^{y})	754.0 (645.0)
AWN-1 (re/mL)	2903^{a}	2391^{ab}	1636^{b}	539.0
OPN-9 (re/mL)	645^{a}	489^{a}	309^{b}	65.0
OPN-12 (re/mL)	645^{a}	503^{a}	348^{b}	68.0
OPN-70 (re/mL)	906^{a}	731^{ab}	515^{b}	113.0

Values in the table are least square means (LSM).
^{xy}: LSM with different superscripts within each row for all nine boars in experiment were different ($P<0.05$).
^{ab}: LSM with different superscripts within each row for four selected boars in experiment were different ($P<0.05$).
± SE: ± pooled standard errors of LSM.
re/mL = relative units per mL of seminal plasma.
PSP-I: Porcine seminal plasma glyoprotein–I ; OPN: Osteopontin

Table 3. Differences between the Highest (R-2, Y-2) and Lowest (G-1, R-1) fertility boars in the seminal plasma proteins of the Sperm-Rich fraction.

Seminal Plasma Proteins	**Fertility ranking**		
	Highest	**Lowest**	**± SE**
Total protein concentration	20	25	5
AWN-1 concentration (re/mL)	1997[a]	3021[b]	397
Total protein (mg/AI dose)	67	94	13
AWN-1 (re/AI dose)	6926[a]	11696[b]	1509
OPN-9 (re/AI dose)	1477[a]	2509[b]	215
OPN-12 (re/AI dose)	1541[a]	2580[b]	242
OPN-70 (re/AI dose)	3971	3708	737
Semen volume (re/AI dose)	3.4	3.79	0.46

Values in the table are least square means (LSM) ± pooled standard errors (SE) of LSM.
[ab]: LSM with different superscripts within each row were different ($P<0.05$).
re/AI dose: relative units per artificial insemination dose.
OPN: Osteopontin.

Table 4. Identification of seminal plasma proteins by liquid chromatography-tandem mass spectrometry.

Spot #	**Protein Name**	**Accession Number**	**Theoretical MW(kDa) /pI**	**Number of unique peptides**	**Protein Coverage (%)**
17	Major Seminal Plasma Glycoprotein (PSP-1)	P35495	14.5/8.33	3	28
22	Major Seminal Plasma Glycoprotein (PSP-1)	P35495	14.5/8.33	3	27.8
26	Epididymal Secretory Gluthatione Peroxidase-5 (GPX)5	O18994	24.9/5.70	10	56
27	Major Seminal Plasma Glycoprotein (PSP-1)	P35495	14.5/8.33	2	17

Figure 1.

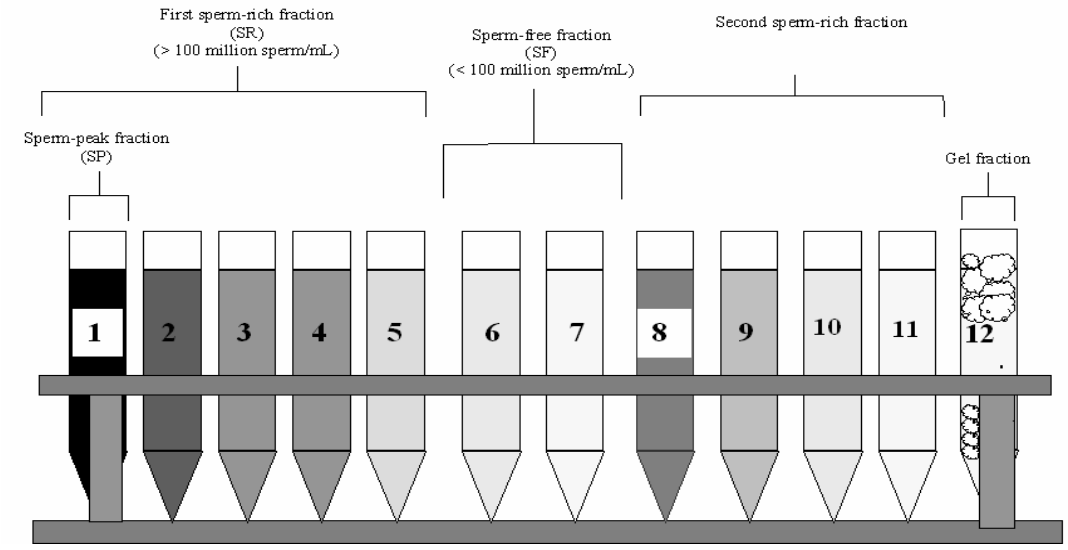
First sperm-rich fraction
(SR)
(> 100 million sperm/mL)
Sperm-free fraction
(SF)
(< 100 million sperm/mL)
Second sperm-rich fraction
Sperm-peak fraction
(SP)
Gel fraction
1
2
3
4
5
6
7
8
9
10
11
12

Figure 2.

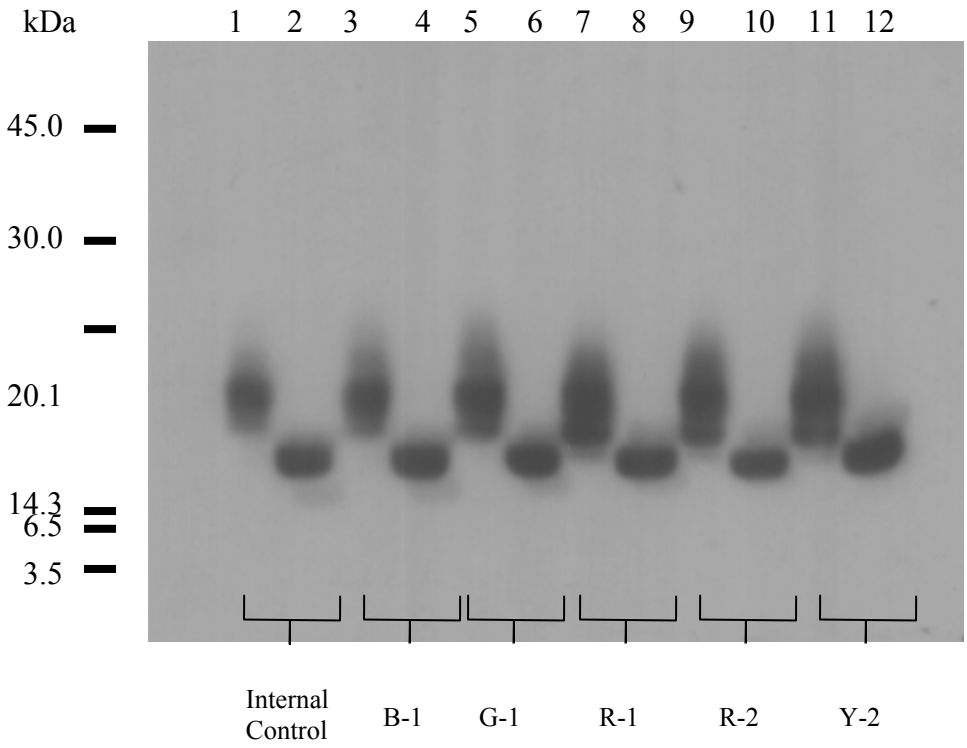
kDa
1 2 3 4 5 6 7 8 9 10 11 12
45.0
30.0
20.1
14.3
6.5
3.5
Internal Control
B-1
G-1
R-1
R-2
Y-2

Figure 3.

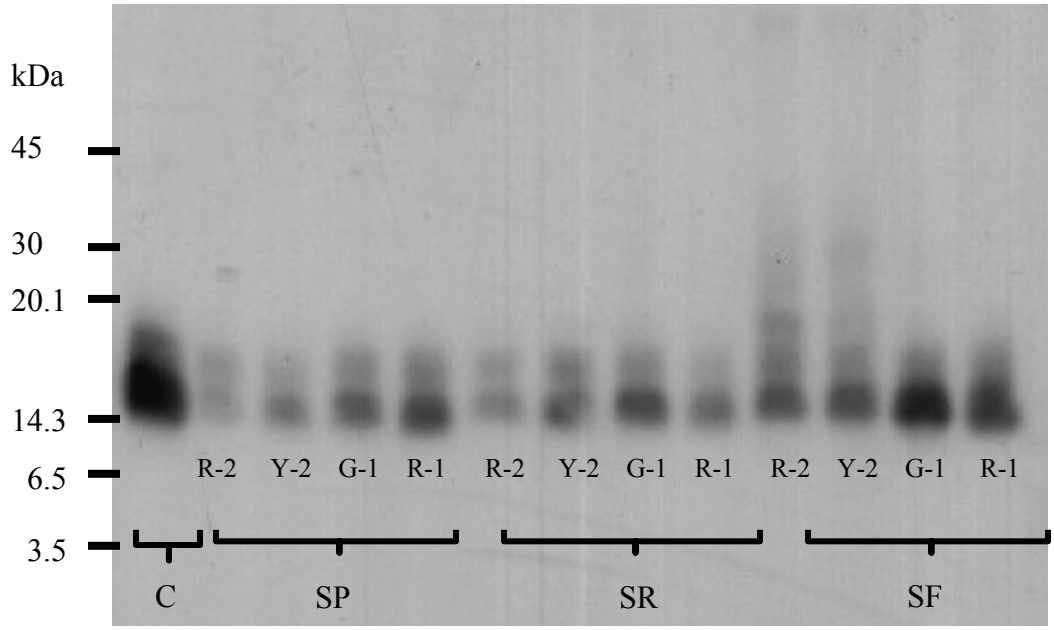

Figure 4.

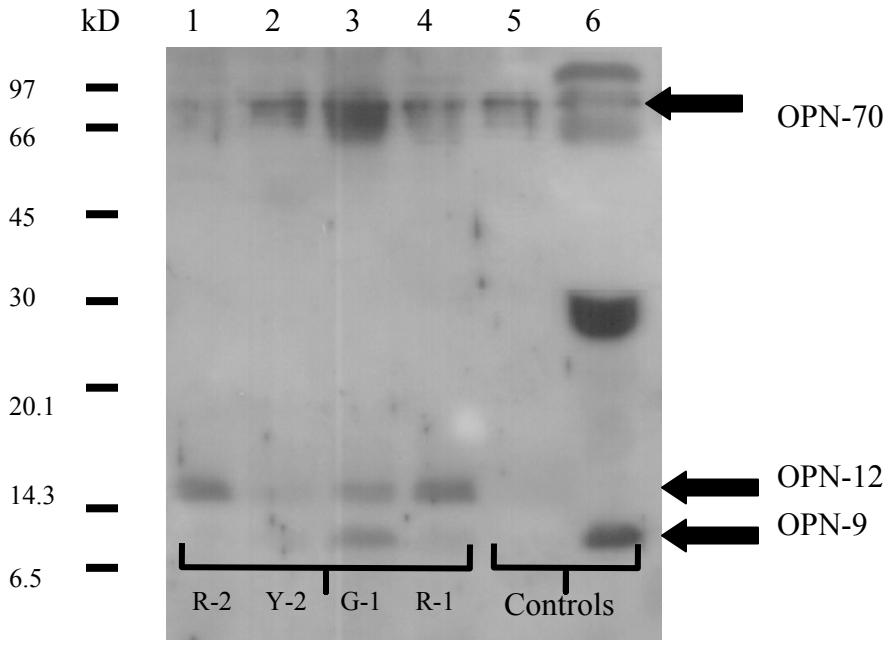
kD
1
2
3
4
5
6
97
66
45
30
20.1
14.3
6.5
OPN-70
OPN-12
OPN-9
R-2
Y-2
G-1
R-1
Controls

Figure 5.