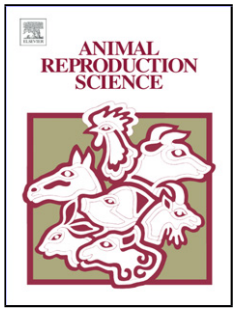

Title: Seminal plasma proteins and their relationship with percentage of morphologically normal sperm in 2-year-old Brahman (*Bos indicus*) bulls

Author: G.B. Boe-Hansen J.P.A. Rego J.M. Crisp A.A. Moura A.S. Nouwens Y. Li B. Venus B.M. Burns M.R. McGowan

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Highlights

1. Fifty-six 2 year old Australian *Bos indicus* Brahman bulls were electroejaulated.
2. Seminal plasma proteins were associated with morphologically normal sperm (PNS24).
3. Proteins with negative and positive relations were identified using mass spectrometry.
4. Six proteins explained 35% of the phenotypic variation in PNS24.
5. Identification of biomarkers related to reproductive performance of beef herds.

Seminal plasma proteins and their relationship with percentage of morphologically normal sperm in 2-year-old Brahman (*Bos indicus*) bulls

Boe-Hansen, G.B.[1*], Rego, J.P.A.[2], Crisp, J.M.[1], Moura, A.A.[2], Nouwens, A.S.[3], Li, Y.[4], Venus, B.[5], Burns, B.M.[6], and McGowan, M.R[1].

[1] School of Veterinary Science, The University of Queensland, Queensland, Australia.

[2] Department of Animal Science, The Federal University of Ceará, Brazil.

[3] School of Chemistry & Molecular Biosciences, The University of Queensland, St Lucia, Queensland, Australia.

[4] CSIRO Agriculture Flagship, St Lucia, Queensland, Australia.

[5] The University of Queensland, Centre for Animal Science, Queensland Alliance for Agriculture & Food Innovation, St Lucia, Queensland, Australia.

[6] Queensland Alliance for Agriculture and Food Innovation, Rockhampton, Queensland, Australia.

*Corresponding author. Tel. +61 0754601857; e-mail address. g.boehansen@uq.edu.au

Keywords

Seminal plasma proteomics, Sperm morphology, Brahman cattle

Abstract

The objective was to determine the relationship between seminal plasma proteins and sperm morphology in *Bos indicus* bulls of the Brahman breed. Fifty-six 24-month-old Australian

Brahman bulls were electroejaculated and samples were examined to determine the percentage of morphologically normal sperm (PNS24) and the seminal plasma protein composition was identified and quantified by 2-D gel electrophoresis. The total integrated optical density of 152 seminal plasma protein spots (SPPs) across all gels was determined using the PDQuest software version 8.0 (Bio Rad, USA). Using a single regression mixed model with the density of individual spots as a covariate for PNS24, 17 SPPs were significantly associated with PNS24 ($p<0.05$). A multiple regression analyses of these SPPs, using three models; non-parametric Tree Model, Generalised Additive Model, and a step-wise selection method was conducted, and 6 SPPs could be used to predict PNS24; four SPPs had positive and two had negative association with PNS24. Together these spots explained 35% of the phenotypic variation in PNS24. Using mass spectrometry (MALDI-ToF and TripleToF-MS) the SPPs with positive relationship contained mainly apolipoprotein A-I (1310), protein DJ-1 and glutathione peroxidase 3 (2308), phosphoglycerate kinase 1 (6402) and apolipoprotein A-I and secretoglobin family 1D member (8008). The SPPs inversely associated with PNS24 were clusterin/seminal plasma protein A3 (1411) and epididymal secretory protein E1 (8108). This is the first comprehensive report on the association between seminal plasma protein composition in *Bos indicus* Brahman bulls and sperm morphology.

Introduction

Traditionally, assessment of male fertility has been focused on examination of the ejaculated sperm. However, evaluating the microenvironment surrounding the sperm cell at the time of ejaculation has potential advantages as it defines the function and health of the genital tract in which the sperm was matured and subsequently transported. It is commonly accepted that seminal plasma proteins play important roles in the physiology and alterations of sperm along both the male and female reproductive tracts. Seminal plasma proteins

contribute to the sperm maturation, metabolism, capacitation, protection, motility, modification of sperm membranes, acrosome reaction, interaction with the oviductal epithelium and fertilization (Rodriguez-Martinez et al., 2011; Caballero et al., 2012). The protein composition of fluids from different parts of the male genital tract of *Bos taurus* bulls, including the epididymis (Moura et al., 2006a; Moura et al., 2010; Belleannee et al., 2011), and accessory sex glands (Moura et al., 2007b) have been described, as well as the composition of ejaculated seminal plasma collected using an artificial vagina (Kelly et al., 2006). In *Bos indicus* bulls, the major components of the seminal plasma, collected using electroejaculation, have been described for Nelore (Assumpcao et al., 2005) and Brahman (Rego et al., 2014) breeds. The studies in the Holstein bull have also identified specific proteins with associations with fertility indexes (Moura, 2005; Moura et al., 2006a; Moura et al., 2006b; Moura et al., 2007a), however meaningful empirical relations between fertility and ejaculated seminal plasma proteins in *Bos indicus* bulls have not been investigated.

Differences between the two subspecies *Bos taurus* and *Bos indicus* in metabolic rate, food and water utilization, sweat glands, skin characteristics and libido have been identified, reflecting adaptation of the *Bos indicus* to the tropical environment and natural pastures (Turner, 1980). *Bos indicus* and crossbred bulls have an increased testicular blood supply compared to *Bos taurus* bulls, likely due to differences in the morphology of the testicular vascular cone. Such attribute improves thermoregulatory capabilities, with positive effects on semen quality and sperm production (Brito et al., 2004; Brito et al., 2012). Moreover, it can be hypothesized that other compensatory mechanisms on the molecular level supporting sperm survival are in place in animals exposed to increased temperatures, perhaps as an effect of physiological, metabolic and endocrine adaptations (Setchell, 1998).

Bull breeding soundness evaluations have been established to include a whole animal approach to determine a bull's reproductive ability, involving a physical evaluation and basic

assessment of semen quality (Kastelic and Thundathil, 2008). The percentage of morphologically normal sperm (PNS) has been correlated to fertility and calf output, particularly in Brahman cattle (Holroyd et al., 2002). A recent genetic study in tropically adapted Australian bulls; Brahman and Tropical Composites also identified semen quality as a major determinant of bull fertility and found that the most heritable measure amongst the semen quality traits studied was the PNS (Corbet et al., 2013). Understanding the physiological functions and relationships between seminal plasma proteins and sperm attributes is an important step toward identifying potential biomarkers for early life prediction of male fertility, and in improving sperm preservation methods. Recently, we have studied the proteome of the seminal plasma of adult *Bos indicus* bulls of the Brahman breed and described the expression of diverse categories of proteins potentially involved in several events, from sperm protection and capacitation to fertilization (Rego et al., 2014). Thus, given such findings, we carried out the present study to determine if significant relationships exist between the expression of proteins in seminal plasma and the percentage of morphologically normal sperm in post-pubertal *Bos indicus* Brahman bulls.

Materials and Methods

Experimental design

The present study represents a continuing effort following a primary work focused on the description of the seminal plasma proteome of Brahman (*Bos indicus*) bulls (Rego et al., 2014). As described therein, fifty-six 24-month old Brahman bulls from the Cooperative Research Centre (CRC) for Beef Genetic Technologies were used in this study. The bulls were bred as part of a large genetic study investigating the relationships between carcass, production traits and fertility traits (Corbet et al., 2013). The bulls were managed from weaning to 2 years of age at either the Brigalow or Belmont Beef Cattle Research Stations

near Theodore and Rockhampton, respectively, in central Queensland, Australia. Initially, semen samples were collected via electroejaculation from 109 bulls and aliquots from each ejaculate were assessed microscopically for sperm mass activity and motility, as reported before (Fordyce et al., 2006). An aliquot was fixed in PBS-glutaraldehyde (0.2%) for the percentage of morphologically normal sperm cells (PNS) under phase contrast microscopy at 1000 x magnification counting 100 sperm per ejaculate (Fordyce et al., 2006) by a Australian Cattle Vets accredited sperm morphologist. From the remaining semen sample, seminal plasma was separated from the sperm by centrifugation at 700 g for 10 min, snap frozen in liquid nitrogen and placed in a -80°C freezer until further analysis.

Seminal plasma protein identification

Total protein concentration was determined in all seminal plasma samples (Bradford, 1976) and samples with less than 12 mg/mL of protein in seminal fluid were not used in the study. Based on this parameter, samples from 56 bulls were chosen and subjected to two-dimensional gel electrophoresis and protein identification by mass spectrometry, as described previously (Rego et al., 2014). In summary, 750 μg of seminal plasma protein was mixed with a re-hydration buffer using 24-cm IPG (immobilized pH gradient) strips (pH 3 to 11, linear; GE Lifesciences, USA). Isoelectric focusing was carried out (Ettan™ IPGphor 2™; GE Lifesciences, USA) according to the following program: 100 V for 2 h, 250V for 2 h, 1000 V for 1 h, 5000 V for 2 h gradient, 5000 V for 8 h and a 30 V hold. After focusing, IPG strips were incubated in equilibration buffers, subjected to SDS-PAGE (15%) in a Caster Ettan Dalt II system (Amersham Pharmacia Biotech, USA) and run at 40 mA per gel. Gels were fixed in methanol and acetic acid and then stained with Sypro Ruby (Life technologies, Invitrogen, USA). The protein gels were then destained and scanned (Typhoon 9400, Amersham Pharmacia Biotech, USA) and evaluated in a single match set (PDQuest software, version 8.0; Bio Rad, USA), as previously reported in detail (Moura et al., 2006b; Moura et

al., 2007b; Souza et al., 2012). Based on PDQuest analysis, protein quantities were estimated as parts per million of the total integrated optical density of spots in the gels.

For mass spectrometry, seminal plasma samples from three bulls (from the group of 56) were subjected to new 2-D electrophoresis, staining gels in colloidal Coomassie blue G-250. Then, spots presenting significant associations with PNS were excised from gels, destained and subjected to in-gel trypsin digestion. Tryptic peptides were then separated using reversed-phase chromatography on a Shimadzu Prominence nanoLC system (Kappler and Nouwens, 2013). Using a flow rate of 30 µL/min, the samples were desalted on an Agilent C18 trap, followed by separation on a Vydac Everest C18 column. A gradient of buffers was used to separate peptides and eluted peptides were analysed on a TripleTof 5600 instrument (ABSciex, USA), with a Nanospray III interface. MS TOF scan across m/z 350-1800 was performed for 0.5 s followed by information dependent acquisition of 20 peptides across m/z 40-1800 (0.05 s per spectra). Data acquired was converted to Mascot Generic Format (mgf) using PeakView v1.1 software (ABSciex, USA) and searched using an onsite version of MASCOT against SwissProt and NCBInr (taxonomy: mammals), accessed via the Australian Proteomics Computational Facility (www.apcf.edu.au). Search parameters were the same as reported in Rego et al. (2014).

Statistical analysis

Prior to fitting the seminal plasma proteins in the models, a Linear Mixed full animal model (i.e. taking pedigree relationship into account) using ASReml_R (Version 3.0) was initially applied to investigate any other significant fixed or random effects affecting the percentage of morphologically normal sperm of the 24-month old bulls (PNS24). With PNS24 treated as a continuous variable, the fixed effects in the model included the variations contributed by sire date of birth, sire origin, dam origin, precise age of bull at the time of

semen collection, and cohort. The random effect included the additive genetic effect of animals. Among all fixed effects, only sire date of birth and sire origin were found to have significant effects on PNS24. Therefore these two fixed effects and additive genetic effect of animals (random effect) were then included in the further analyses. Both linear and non-linear relationships between seminal plasma protein spots and PNS24 were then examined using single and multiple regression models, respectively. For a single regression model, an individual seminal plasma protein spot (SPP) was fitted as a covariate.

The statistical model was as follow:

PNS24 =μ + sire dob + sire origin + animal + LogSPP (or SQRTSPP) + error

where μ is the population mean, sire dob is sire date of birth, LogSPP (or SQRTSPP) refers to the $\log_{10}$ transformed or squared root transformed protein data.

Since single regression analysis ignored the relations between SPPs, further multiple regression analyses were conducted to detect any potential interactions among SPPs as well as curvature relationships between SPP and PNS24. After individual significant SPPs were identified from the single regression analyses, their combined effects were then assessed with three multiple regression models. Two non-parametric multiple regression models in R program (Version 2.13.1), namely Tree Model (Tree, (Breiman et al., 1984) and Generalised Additive Model (GAM, (Hastie and Tibshirani, 1990), were used to detect potential interactions among SPPs and curvature relationships between SPPs and PNS24. Based on the results from Tree and GAM models, a step-wise selection method was then used in a generalised multiple regression model to derive the parameters for final SPP predictors for PNS24.

Protein identification

Although the major seminal plasma proteins of the Brahman bulls used in the present study had been described in the companion publication (Rego et al., 2014), spots that presented significant statistical relationships with PNS24 were subjected to a new identification by mass spectrometry. For this purpose, seminal plasma samples from six bulls (chosen from the group of 56 animals) were subjected to 2-D SDS-PAGE, staining gels with Coomassie blue. Then, the spots of interest were excised from the gels, destained and subjected to in-gel protein digestion and Zip-tip clean up. Mass spectrometry (ESI-qTOF) and data search for protein identification were also carried out as described (Rego et al., 2014).

Results

Semen criteria and 2-D gels

Based on the analysis of the semen samples from all 56 bulls, the average percentages of forward progressively motile sperm and normal sperm were 70.4 ± 2.3 % and 64 ± 3.2 %, respectively (Table 1; as previously reported by (Rego et al., 2014)). The distribution of PNS at 24 months in the group of 56 bulls reflected that observed in the cohort of the original 109 bulls (Figure 1). A master gel representing the seminal plasma proteins of the Brahman bulls is shown in Figure 2 as well as the four gels used for spot excision and protein identification are presented in Supplemental Figure 1. The master gel and 17 of the 2-D seminal plasma maps have been described (Rego et al., 2014).

Single and multiple regression analyses

Single regression analysis using either Logarithmic or squared root transformation of spot intensities identified 17 spots with significant associations ($P < 0.05$) with PNS24 (Table 2). Among them, 13 spots were common in both methods (Table 2). The analysis of these 13 spots with the non-parametric Tree model regardless of log or squared root transformation of data, revealed a very complex hierarchical relationship among several proteins. Figure 3 illustrates the relationships between 6 SPPs (log-transformed) and PNS24. Protein spot 1411 was found to be the most important SPP affecting PNS24, followed by spots 1310, 8108 and 2308, and then spots 8008 and 6402 (Figure 3). Selecting the animals with log(1411) < 6.87, log(1310) > 6.7 and log(2308) > 5.58 would expect to increase PNS24 to 83.3% (Figure 3). The same four seminal plasma protein spots (spot 1411, 1310, 8108 and 2308) were also identified by the Tree model using the squared root transformation (not shown).

The analyses using Generalized Additive Model (GAM) on the proteins from Tree models showed non-linear curvature relationships between these proteins and PNS24 (Figure 4). The GAM models confirmed spots 1310, 1411 and 8008 in log-transformation (Figure 4) and 1411 from squared root transformation (not shown) having significant effects on PNS24, respectively ($p<0.05$). The corresponding R^2 value was 65% in log-transformation and 56% in squared root transformation.

Based on the results from the Tree and GAM models, a refined multiple regression model with linear terms of significant SPPs and their two-way interactions was further examined. A step-wise selection model showed that with log-transformation, the six protein spots (spot 1310, 1411, 2308, 6402, 8008 and 8108) could be used to predict the PNS24 (Table 3). Among these, four of the spots (spots 1310, 2308, 6402 and 8008) had a positive association and the remaining two spots (spots 1411 and 8108) had a negative relation with PNS24 (Table 3 and Figure 3). Together, all of these spots explained 35% of the phenotypic

variation in PNS24, 53% when also including two other fixed effects, sire origin and date of birth, in the model (Table 3). With squared-root transformation, the step-wise selection model identified the same 3 SPPs (spot 1310, 1411 and 2308) and one new SPP (spot 4). Together, the intensity of these spots explained 32% of phenotypic variation in PNS24 (not shown).

Confirmation of protein identity

Identity of the six spots shown to be significantly associated with the percentage of normal sperm in the ejaculates of 24-month old bulls was confirmed by mass spectrometry (Table 4; Figure 2; Supplemental Figure 1). Four of these spots had positive relationship with PNS24 and contained mainly apolipoprotein A-I (spot 1310), protein DJ-1/glutathione peroxidase (spot 2308), phosphoglycerate kinase 1 (spot 6402) and apolipoprotein A-I/secretoglobin family 1D member (spot 8008). Spots inversely associated with PNS24 were identified mainly as clusterin and seminal plasma protein A3 (spot 1411) and epididymal secretory protein E1 (spot 8108).

Discussion

The present study describes empirical relationships between seminal plasma proteins and the percentage of morphologically normal sperm in ejaculates of 24-month old Brahman bulls, using a 2-D gel-based proteomic approach and comprehensive statistical analysis models. In addition to the conventional single SPP regression model that only examines a linear function between a SPP and PNS24, we applied three multiple regression statistical models to assess complex relationships among SPPs as well as these SPPs with PNS24. Two non-parametric multiple variable models, Tree and GAM, are usually used for the cases when there is no prior knowledge on suitable mathematic functions to choose from. The Tree model performs recursively partition of multiple predictors into subsets (Breiman et al., 1984)

and can reveal hierarchical relationships among predictors and a response variable. In addition, they have an advantage in dealing with dataset issues such as the number of predictors is much larger than the number of samples, as the limitation of our data here. GAM model takes each predictor variable in the model and separates it into sections and then fits polynomial functions to each section (Hastie and Tibshirani, 1990). As a result, GAM produces a smoothing spline function. From the results, it is clear that the conventional single SPP (e.g. Log-transformed) regression model identified 17 spots significantly associating with PNS24. However, with the limited population size of 56 animals, it is impossible for a conventional parametric statistical model to simultaneously fit all these SPPs in a single model to examine all possible relationships between SPPs and PNS24. In this study, both Tree and GAM models provided crucial information on identifying subset of significant SPPs and their curvature relationships with PNS24. Finally, the step-wise multiple regression model was used for a final prediction function. Out of 152 SPPs, we identified the integrated optical density of six protein spots to be significantly associated with PNS24 evaluated in 56 bulls with reproductive status that are representative of the average population of Brahman cattle raised in Northern Australia (Fitzpatrick et al., 2002). The identity of those six spots, included in a companion publication about the seminal plasma proteome of electroejaculated *Bos indicus* Brahman bulls (Rego et al., 2014), was presently confirmed by a second mass spectrometry analysis. The likely roles of each of these seminal fluid proteins are discussed below.

Apolipoprotein AI (ApoA1) is lipophilic and potentially involved in sperm membrane modulation and capacitation. Apo1 has been found in epididymal fluid from Holstein (Moura et al., 2010) and Charolais bulls (Belleannee et al., 2011) and from vesicular gland fluid and seminal plasma from tropically adapted rams (Souza et al., 2012). ApoA1 is part of the high-density lipoprotein (HDL) complex. ApoA1 may interact with a protein located on the

flagellum and in the acrosome of mouse sperm (Jha et al., 2008) and induce HDL-mediated lipid transport and cholesterol efflux, events linked to sperm capacitation (Therien et al., 1998). In the current study, ApoA1 was present in spots 1310 and 8008, both positively associated with PNS24, but also in spot 1411, which was negatively associated with PNS24. However, the MS score and sequence covered was much lower for ApoA1 in spot 1411 than in the two other spots. Clusterin was found in significant amounts in spot 1411. The high sensitivity of the mass spectrometry method used in the current study is likely a contributing factor to this finding.

A spot containing Protein DJ-1 (PDJ-1) (spot 2308) was positively associated with PNS24. PDJ-1 is found in sperm, testes and epididymides of humans (Yoshida et al., 2003) and epididymal fluid of bulls (Belleannee et al., 2011). PDJ-1's role is similar to that of glutathione peroxidase (Lev et al., 2009; Yu et al., 2013) acting as a redox-sensitive chaperone and as a sensor for oxidative stress, as it is transcribed in response to cellular damage caused by oxidative stress. The sperm membrane protein SP22, also referred to as CAP1, Protein DJ-1, RS and PARK7 (Klinefelter, 2008), correlates with fertility, especially in toxicological studies using rodents and rabbits (Klinefelter et al., 1997; Klinefelter et al., 2002; Kaydos et al., 2004; Veeramachaneni et al., 2007). PDJ-1 binds to the equatorial segment and tail of rodent sperm, suggesting a role in either sperm-egg interaction, sperm motility, or both (Klinefelter et al., 2002). The value of this protein as a biomarker with consistent effect on fertility has previously been acknowledged (Klinefelter, 2008). In fact, sperm PDJ-1 is reduced in asthenozoospermic men and its concentration was positively correlated with sperm motility and sperm superoxide dismutase activity in these patients as well (An et al., 2011). In a study carried out in tropically adapted rams, sperm membrane SP22 presented negative correlations with the percentage of morphologically abnormal sperm and positive with that of sperm with intact membranes (Favareto et al., 2010). Thus, there is

evidence that PDJ-1 is a marker of fertility in other species but this is apparently the first report of its associations with sperm parameters of bulls. Differences in the type of associations involving this protein, either positive or negative, depend on the species and variables used as endpoints in the statistical analysis.

Glutathione peroxidase 3 was found in spot 2803, along with PDJ-1, and to a much lesser extend in spot 1310, which also contained large amounts of ApoA1. The intensities of these two spots were positively associated with PNS24. Glutathione peroxidase 3 is referred to as either an extracellular or plasma-type peroxidase, and is expressed in several tissues, including the male genital tract. Different isoforms of glutathione peroxidase have been detected in caudal epididymal fluid of Holstein bulls (Moura et al., 2010), and in both epididymis and vas deferens of mice (Schwaab et al., 1998). Like clusterin, glutathione peroxidase 5 is up regulated in pyriform sperm from scrotal-insulated *Bos taurus* bulls (Shojaei Saadi et al., 2013). In a recent review on mammalian glutathione peroxidases, the importance of such protein type for sperm integrity and fertility is highlighted (Chabory et al., 2010), but there is still limited knowledge about glutathione peroxidase 3 as an indicator of normal spermatogenesis and sperm morphology in the bull.

The intensity of a spot containing phosphoglycerate kinase 1 was positively related to PNS24. A protein similar to testis specific phosphoglycerate kinase has been identified in seminal plasma of *Bos taurus* bulls (Kelly et al., 2006) and the isoenzyme, phosphoglycerate kinase 2, in epididymal fluid from Charolais bulls (Belleannee et al., 2011). However, phosphoglycerate kinase 1 has been found in seminal plasma, but not caudal epididymal fluid in tropically-adapted rams (Souza et al., 2012) and in proteomic analysis of human prostasomes (Utleg et al., 2003). Phosphoglycerate kinase is the first enzyme in the glycolytic pathway that generates ATP and is positively associated with sperm motility in men (Schirren et al., 1979). A study have also shown that *Pgk2-/-* mice have normal testis histology, sperm

count and sperm ultrastructure, but decreased sperm metabolism and motility (Danshina et al., 2010), illustrating that the glycolytic pathway is important for gamete function and fertility.

Secretoglobin 1D was found in spot 8008, along with ApoA1, and positively associated with PNS24. Secretoglobin 1D, also known as lipophilin-A, is a member of the uteroglobin family. Uteroglobin-like protein can be detected in the uterus, seminal vesicle secretion and seminal plasma of rabbits (Beier et al., 1975). In humans, uteroglobin from accessory sex gland fluid binds to ejaculated sperm, with capacitation-inhibiting activity and a negative effect on sperm motility (Luconi et al., 2000). Secretoglobin 1D had never been described before in seminal plasma of bulls, until the recent companion publication using the same group of animals; (Rego et al., 2014). Thus, functions of this protein and how it possibly affects male fertility in the bovine are still unknown and needs further investigation.

A form of seminal plasma clusterin (spot 1411) was inversely related to PNS24. This spot also contained seminal plasma protein A3 (BSP A3; (Manjunath et al., 2009)) and ApoA1, but the MS/MS protein score associated with clusterin identification was much higher than for the other proteins. Clusterin binds to cells, membranes and hydrophobic proteins and several of its isoforms have been detected in the male genital tract including the rete testis fluid, cauda epididymal fluid and sperm (Ibrahim et al., 1999; Moura et al., 2010), accessory sex gland fluid (Moura et al., 2007b) and seminal plasma of bulls (Kelly et al., 2006).

The function of clusterin in the male reproductive tract varies and depends on its tertiary structure, posttranslational modifications, origin and location of the protein (Sensibar et al., 1993). Clusterin is involved in sperm protection and is secreted in response to cellular damage and heat-shock (Bailey and Griswold, 1999), and acts like a chaperone, shielding cells against protein precipitation and complement-mediated attack by the immune system

(Bailey et al., 2002). In men, clusterin helps with removal of defective sperm in the epididymis (Zalata et al., 2012). Scrotal insulation in rams (Ibrahim et al., 2001b) and bulls (Shojaei Saadi et al., 2013) increases the number of clusterin positive sperm and morphologically abnormal sperm, suggesting that this protein is an indicator of poor semen quality. Bulls with a high percentage of morphological abnormal sperm, lower sperm motility and lower fertility rates have been shown to have a greater number of clusterin positive sperm cells (Ibrahim et al., 2000; Ibrahim et al., 2001a). Furthermore, a study showed an association between abnormal sperm head morphology (pyriform sperm) after scrotal insulation and an increase in clusterin expression on the sperm membrane in Holstein bulls (Shojaei Saadi et al., 2013). In stallions, a negative correlation between a 52-kDa clusterin isoform and first cycle conception rate was found (Novak et al., 2010) and in men the expression of a 40-kDa clusterin isoform was significantly increased in semen from infertile men (Zalata et al., 2012). More recently, a study reported a negative correlation between the intensity of a clusterin spot in 2-D gels of peccary seminal plasma and the percentage of sperm with functional membrane (Santos et al., 2014). In general, several studies seem to agree with our current investigation, indicating that high levels of clusterin in seminal plasma and sperm may reflect a deviation from normal spermatogenesis.

Seminal plasma protein A3 (currently known as BSP) belongs to the Binder of SPerm protein (BSP) family, a group of molecules with phospholipid binding properties (Manjunath et al., 2009). BSP A3 was found in spot 1411, which also contained clusterin and small amounts of ApoA1. Studies have pointed out that BSPs interact with molecules such as ApoA1 and with HDL (Manjunath et al., 1989; Desnoyers and Manjunath, 1992), which explains why we detected BSP A3 and ApoA1 in a same spot. Given this interaction, it is also possible that free ApoA1, present in the two spots positively related to PNS24 (spot 1310 and 8008), affect sperm cells or reflect the condition of the sperm, differently from when

ApoA1 is linked with BSP A3 and clusterin, which was found in the spot with a strong inverse relation to PNS24 (spot 1411). In ruminants, BSPs regulate phospholipid and cholesterol efflux from sperm membrane and sperm capacitation (Manjunath and Therien, 2002), as well as interactions between sperm and the oviduct (Gwathmey et al., 2006). BSPs are either beneficial or detrimental to sperm, depending on concentrations and exposure (Therien et al., 1998; Manjunath and Therien, 2002). In this regard, a study has demonstrated a positive effect of fluid from the accessory sex glands from high fertility bulls on epididymal sperm from low fertility bulls and one of the proteins associated with such effect was identified as BSP A3 (Moura et al., 2007a). Conversely, BSP A3 is higher on sperm membrane of Nelore bulls defined as less fertile (Roncoletta et al., 2006) and BPS A3 appears down regulated in pyriform sperm obtained after scrotal insulation of Holstein bulls (Shojaei Saadi et al., 2013). Seminal plasma BSP 30kDa, a member of the BSP family, presents a quadratic association with fertility of dairy bulls (Moura et al., 2006a) suggesting that, in fact, high abundance of BSPs may be detrimental to fertility, supported by the empirical associations between BSP A3 and PNS24 presently reported.

Epididymal secretory protein E1 is lipophilic and an abundant protein in the cauda epididymal fluid of Holstein bulls (Moura et al., 2010) and it is present in seminal plasma from both *Bos taurus* and *Bos indicus* (Assumpcao et al., 2005) bulls and tropically-adapted rams (Souza et al., 2012). This molecule attaches to the surface of intact sperm and has been linked to the positive effect of seminal plasma on motility of ram sperm subjected to cryopreservation (Bernardini et al., 2011). In our study epididymal secretory protein E1 (spot 8108) was negatively related to PNS24, different from the results obtained with rams. Caution is needed when comparing results across species, as differences in sperm physiology and structures may exists. The negative correlation between epididymal secretory protein E1 and PNS24 in the Brahman bulls remains to be fully understood.

Metalloproteinase inhibitor 2 (TIMP-2) was also found in spot 8108 with epididymal secretory protein E1. The presence of TIMP-2 in the seminal plasma of Holstein bulls was first reported more than 15 years ago (Calvete et al., 1996; Mortarino et al., 1998) and a positive association between TIMP-2 and fertility in the bull was suggested (McCauley et al., 2001). In our study, the amount of TIMP-2 in spot 8108 is much lower than that of epididymal secretory protein E1 and this may reduce the importance of the associations involving TIMP-2 and PNS24. Nevertheless, further investigation of the physiological mechanism by which TIMP-2 expression appears to be inversely related to the percentage of normal sperm in the ejaculates of Brahman bulls is required.

Conclusions

Specific seminal plasma proteins from Brahman bulls were found to be associated with the percentage of morphologically normal sperm. Such relationships indicate that seminal plasma protein composition in post pubertal bulls represents impressions of testicular and epididymal function or homeostasis of the male genital tract. The dependent variable used in our regression models (PNS24) has previously been shown to be an indicator, from the male side, of calf output. Thus, the empirical associations presently described will have potential consequences on reproductive performance and profitability of beef herds. However, future studies on a large population are need to validate the robustness of these biomarkers by selecting bulls based on the seminal plasma content of those six proteins and further confirming their association with semen quality and fertility.

Conflicts of Interest: none

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Table 1. Descriptive statistics for the 56 Brahman bulls at 24 months of age, given as mean and standard deviation (SD), minimum (min) and maximum (max).

Trait	**Mean (±SD)**	**Min**	**Max**
Body weight (kg)	353 ± 4.9	272	432
Scrotal circumference (cm)	29 ± 0.3	24	34
Progressive motility (%)	70 ± 2.3	30	95
Morphological normal sperm (PNS%)	64 ± 3.2	6	94
Seminal plasma protein conc. (mg/mL)	50.6 ± 1.8	12.0	101.3

Table 2. Significant seminal plasma protein spot (SPP) identified from single regression analyses and their estimated regression coefficients. Seminal plasma samples from electroejaculated *Bos indicus* Brahman bulls at 24 months of age (n = 56). Stderr – standard error, p.vale – P value.

	Log transformation			Square root transformation		
SPP Name	Regression	Stderr	p.val	Regression	Stderr	p.val
4	-2.49	1.22	0.0413	-0.32	0.13	0.0127
1309	3.16	0.71	0.0000	0.30	0.07	0.0000
1310	2.59	0.75	0.0006	0.12	0.05	0.0274
1411	-2.29	0.98	0.0191	-0.67	0.20	0.0009
2308	2.36	1.10	0.0317	0.67	0.28	0.0162
4502	-4.37	1.85	0.0180	-0.50	0.24	0.0364
4504				-0.57	0.27	0.0343
4612	-3.89	1.62	0.0167	-1.18	0.44	0.0078
4802	-2.55	1.20	0.0345	-0.38	0.18	0.0392
5301	-2.31	1.05	0.0277	-0.52	0.25	0.0395
5502	-5.15	2.04	0.0118	-0.77	0.28	0.0057

5802				-0.35	0.17	0.0423
6402	3.47	1.21	0.0041			
8008	-1.96	0.97	0.0441			
8108	-2.30	1.05	0.0292	-0.27	0.12	0.0216
8304	-2.00	0.80	0.0127	-0.20	0.10	0.0455
8305	-1.92	0.82	0.0190	-0.23	0.11	0.0315

Table 3. Final seminal plasma protein spot SPPs (log-transformed) and other factors identified by the multiple regression model with a step-wide selection method, and their estimated effects on the percentage of morphologically normal sperm (PNS24). Seminal plasma samples from electroejaculated *Bos indicus* Brahman bulls at 24 months of age (n = 56). R^2= 0.53, model p-value = 0.00002818.

Model terms	**Estimate**	**Std.**	**Pr(>|t|)**
(Intercept)	-12760.00	3952.00	0.00232
Siredob	0.35	0.11	0.00223
Sireorigin -Tartrus	-244.20	76.09	0.00245
Sireorigin -Weetalaba	-260.80	76.42	0.00137
log(sp1310)	1.76	0.72	0.01885
log(sp1411)	-1.91	0.87	0.03287
log(sp2308)	1.26	0.98	0.20519
log(sp6402)	3.04	1.14	0.01049
log(sp8008)	0.03	0.98	0.97591
log(sp8108)	-1.27	1.01	0.21538

Table 4. Seminal plasma proteins spots (SPPs), of *Bos indicus* Brahman bulls associated with percentage of morphologically normal sperm at 24 month of age (PNS24). Proteins were identified by two-dimensional electrophoresis and tandem mass spectrometry. Spot numbers refer to those shown in the two-dimensional gels of Figure 2.

Spot number and proteins identified	**Experimental kDa/pI**	**Accession number GI**	**MS/MS protein score**
Positive effect on PNS24			
Spot 1310			
Apolipoprotein A-I (*Bos taurus*)	32.2/4.6	APOA1_BOVIN	18564
Glutathione peroxidase 3 (*Bos taurus*)	32.2/4.6	GPX3_BOVIN	43
Spot 2308			
Protein DJ-1 (*Bos taurus*)	25.8/6.1	PARK7_BOVIN	1331
Glutathione peroxidase 3 (*Bos taurus*)	25.6/6.1	GPX3_BOVIN	494
Spot 6402			
Phosphoglycerate kinase 1 (*Bos taurus*)	46.6/8.9	PGK1_BOVIN	2649

Spot 8008			
Apolipoprotein A-I (*Bos taurus*)	10.7/9.2	APOA1_BOVIN	1537
Secretoglobin family 1D member (*Bos taurus*)			
10.7/9.2 SG1D_BOVIN			
986 19			
Negative effect on PNS24			
Spot 1411			
Clusterin (*Bos taurus*)	41.3/4.7	CLUS_BOVIN	2795
Seminal plasma protein A3 (*Bos taurus*)	41.3/4.7	SFP3_BOVIN	404
Apolipoprotein A-I (*Bos taurus*)	41.3/4.7	APOA1_BOVIN	180
Spot 8108			
Epididymal secretory protein E1 (*Bos taurus*)	15.1/9.2	NPC2_BOVIN	3711
Metalloproteinase inhibitor 2 precursor (*Bos taurus*)			
15.1/9.2 TIMP_BOVIN			
102 6			

Figure 1. Distribution of the percentage of morphologically normal sperm at 24 month of age (PNS24) in selected Brahman bulls (n=56) and their cohort (n=109). The mean PNS24 was 64% for the 56 bulls.

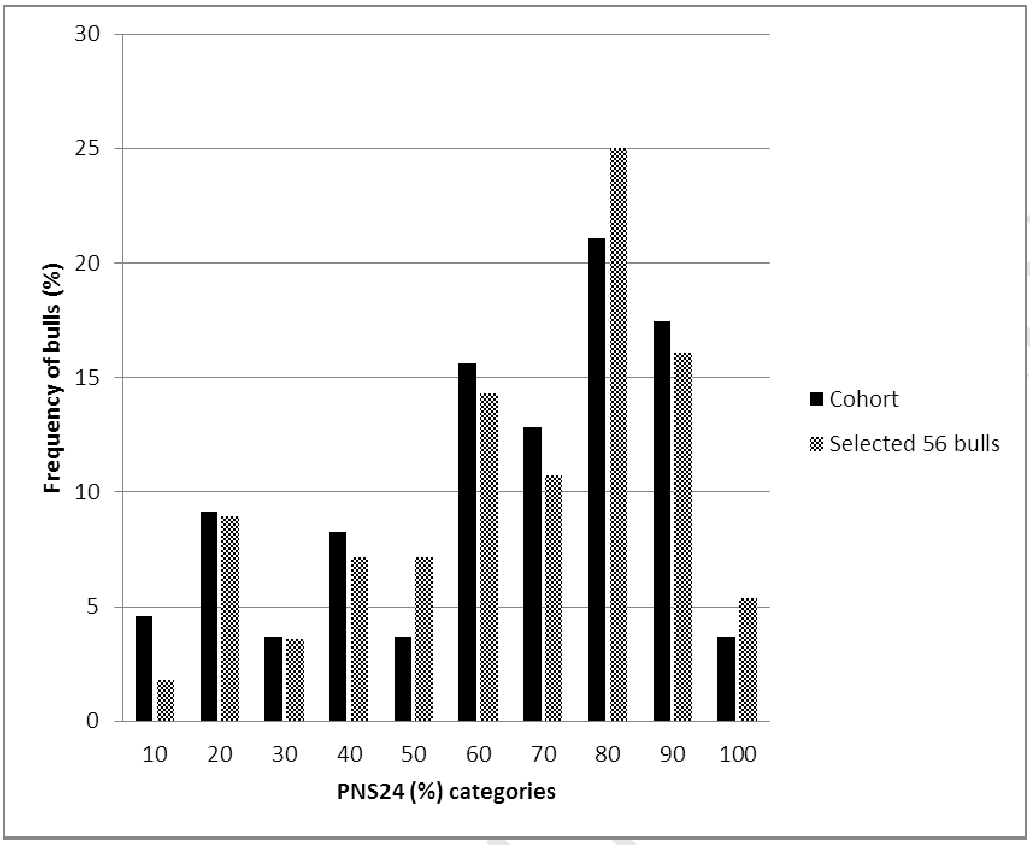

Figure 2. Two-dimensional master map of seminal plasma proteins from selected Brahman bulls. The figure represents the master gel generated by PDQuest software (Version 8.0 Bio Rad, USA), based on a match set with all the gels used in the study. Proteins were stained with Sypro Ruby and identified by two-dimensional electrophoresis and tandem mass spectrometry (ESI-QUAD-ToF). The seminal plasma protein spots correlated with percentage of normal sperm at 24 month of age (PNS24) are identified by numbers and arrows. Spot numbers refer to those listed in Table 2.

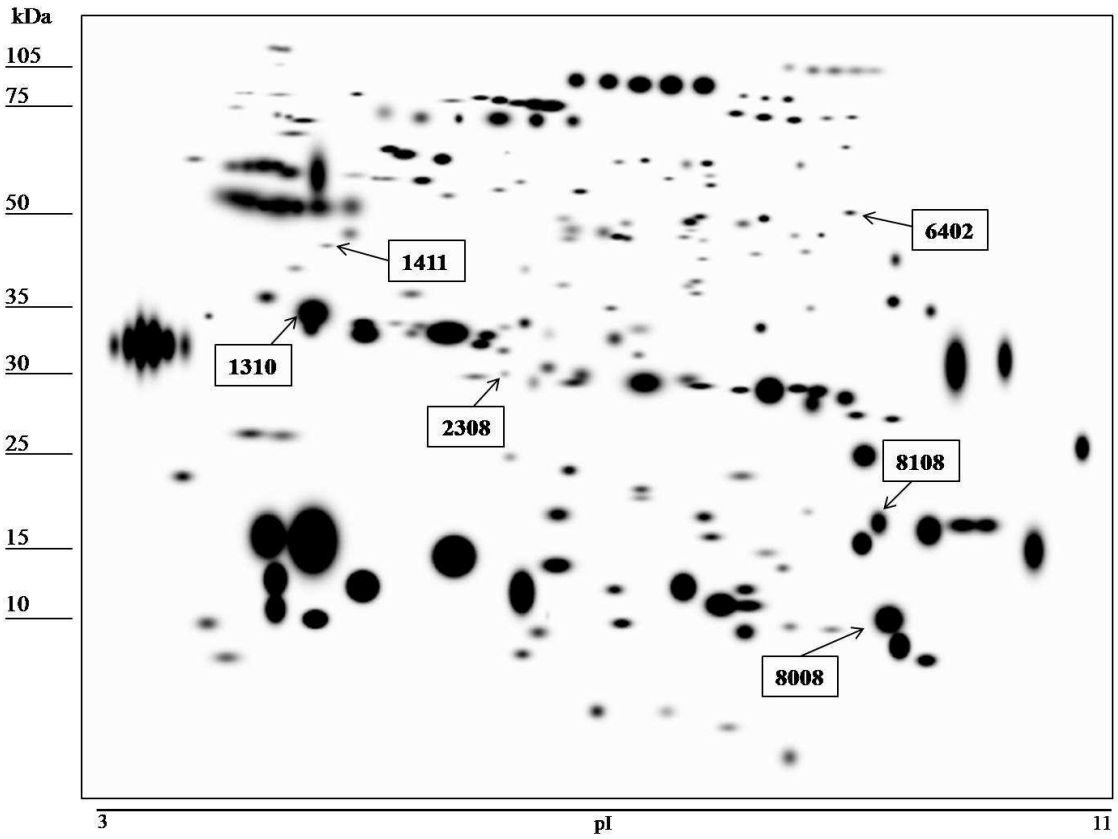

Figure 3. Tree model results from log transformed seminal plasma protein (SPP) intensity data, showing the hierarchical relationships among 6 SPP and percentage of morphologically normal sperm at 24 months of age (PNS24) in electroejaculated *Bos indicus*, Brahman bulls. Spot numbers refer to those shown in the two-dimensional gels of Figure 2. The SPP importance is indicated by the length of the branches. The longer the branches in the tree, the greater the deviance explained. The values in the end of branches refer to the predicted mean values of PNS24.