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Heritability and role of new sperm assays

The use of flow cytometric assays to evaluate sperm chromatin integrity and protamine content.

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Abstract

Heritability and genetics of sperm chromatin integrity and protamine deficiency were studied in Brahman (n = 592) and Tropical Composites (n = 538) bulls using flow cytometry. Frozen sperm samples and fertility phenotypes of Beef CRC bulls were used. Sperm chromatin integrity was measured by the sperm chromatin structure assay (SCSA) and reported as two DNA fragmentation indexes (DFI3 and DFI4). Sperm protamine deficiency was measured using the newly developed sperm protamine deficiency assay (SPDA) and reported as low, medium or high CMA3 binding levels (LCB, MCB and HCB). Heritabilities estimated for these sperm phenotypes using genomics ranged from 0.21 to 0.40. The genetic correlations between DFI3, DFI4 and PNS were estimated as -0.20 and -0.43 in Brahman bulls and -0.32 and -0.36 in Tropical Composites. The genetic correlations between sperm protamine deficiency and percentage of normal sperm (PNS) were estimated as -0.10 (HCB) and -0.22 (MCB) in Brahmans. In Tropical Composites, correlations with PNS were -0.26 (HCB) and -0.34 (MCB). Sperm chromatin integrity and protamine deficiency were genetically correlated traits (r^2 ~ 0.33 to 0.51) in both breeds. Genome-wide studies identified regions of chromosome X that were associated with sperm chromatin integrity, protamine deficiency and PNS.

Executive summary

Bull Breeding Soundness Evaluation (BBSE) is a standard industry practice that enables farmers to identify and purchase bulls with greater fertility potential and discard bulls that would be unfit for mating. In the context of evaluating bulls for breeding soundness, two flow cytometry assays that assess sperm chromatin structure phenotypes were evaluated: sperm DNA susceptibility to fragmentation (also called sperm chromatin integrity) and sperm protamine deficiency.

In the absence of a field trial and fertility data, the percentage of normal sperm (PNS) from morphological analyses was used as an indicator of bull fertility. Previous research demonstrated that PNS was the best predictor of calf output per bull in a multiple-sire mating system (Holroyd *et al.* 2002). In this context, PNS served as basis to infer the association between the two sperm chromatin phenotypes and bull fertility. The present study included semen samples from two types of Australian cattle: Brahman (n = 592) and Tropical Composites (n = 538) previously sampled as part of the Beef CRC, at approximately 24 months of age. These 2 year old bulls were capable of producing sperm samples when electro-ejaculated and were deemed “adult” bulls. Sperm chromatin integrity was measured by the sperm chromatin structure assay (SCSA) and reported as two DNA fragmentation indexes (DFI3 and DFI4). Sperm protamine deficiency was measured using the newly developed sperm protamine deficiency assay (SPDA) and reported as low, medium or high CMA3 binding levels (LCB, MCB and HCB). Both medium and high CMA3 binding levels are indicative of protamine deficiency.

The genetic correlation between DNA fragmentation index (DFI) and PNS was estimated as -0.20 to -0.43 in Brahman bulls. In Tropical Composite bulls, this correlation ranged between -0.32 and -0.36. The genetic correlation between sperm protamine deficiency and PNS was estimated as -0.10 to -0.22 in Brahman and -0.26 to -0.34 in Tropical Composites. In simple terms, higher sperm DNA fragmentation and higher sperm protamine deficiency were associated with higher percentages of sperm abnormalities, which is indicative of lower fertility. The correlations reported herein indicate that sperm chromatin structure integrity and sperm protamine content contribute to overall fertility in tropically adapted bulls. Since existing field fertility research for sperm chromatin structure has only been conducted in dairy bulls, and only for DFI, it is important to further confirm DFI and sperm protamine deficiency as indicators of bull fertility. Consequently further fertility trials are warranted.

The sperm samples from previously genotyped bulls were key to the genetic component of this research. These samples were from bulls that have known pedigree and were genotyped using the Illumina SNP chip technology; over 50,000 genetic markers were tested. These genetic markers are single nucleotide polymorphisms detected in the DNA of tested bulls. The availability of genotypes and pedigrees made it cost effective to estimate heritabilities, perform genome-wide association studies (GWAS) and discover genes associated with sperm chromatin structure traits. This gene discovery exercise reported herein contributes to a better understanding of bovine spermatology and bull fertility. Regions of the X chromosome that are associated with PNS and sperm chromatin phenotypes (DFI3, DFI4, HCB and MCB) were identified. Information gathered in the GWAS may also contribute to genomic selection of fertile bulls or to the identification of less fertile bulls that should be removed from the breeding herd.

In summary, research that can help guide the industry on the relevance of incorporating two flow cytometry assays, the SCSA and the SPDA, in the evaluation

of bull breeding soundness is presented. The evaluation of bull fertility is important in three contexts: 1) the performance of a BBSE (i.e. before the mating season), 2) sperm evaluation prior to crysopreservation and storage of semen for artificial insemination, and 3) in genetic selection programs. SCSA and SPDA were compared with traditional BBSE evaluation of semen quality and found correlations that confirm SCSA and SPDA as potential indicators of bull fertility. Further research is needed to demonstrate the direct effect of SCSA and SPDA in the fertility of tropically adapted bulls. Based on these findings, it is recommended that future trials should include the verification of these phenotypes in an *in vitro* fertilization model and subsequent measurement of field fertility as calf output per bull in a multiple-sire system in tropically adapted bulls. The overall aim would be to determine if DFI and sperm protamine deficiency could be used as predictors of calf output in combination with other fertility indicators, used in current BBSE.

In summary, sperm chromatin phenotypes are heritable and hold potential as indicators of bull fertility. For further development of genomic tools targeting bull fertility, an in-depth analyses of chromosome X mutations is recommended. Future research could also explore SCSA and SPDA in *in vitro* and *in vivo* fertility trials to further examine their potential as indicators for bull fertility.

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1. Background

Bull Breeding Soundness Evaluation (BBSE) is a standard industry practice that enables farmers to identify and purchase potential fertile bulls and discard bulls that would be unfit for breeding. The use of BBSE as a mean to improve herd fertility is highly important. A 50% failure of bulls that undergo a BBSE test only three months after sale was reported and related to relocation and management of bulls post-sale (Holroyd et al. 2004). The currently used BBSE in Australia has been standardized and is based on the measurement of traits grouped into five categories: scrotum, physical, crush-side semen evaluation, sperm morphology, and serving (Fordyce et al. 2006). All categories are important as together they form a robust assessment enabling an evaluation of the bulls mating and fertility capacity. It is imperative that revisions and improvements to any aspects of the BBSE should be based on scientific evidence.

For this project, \ two categories of the BBSE were examined: crush-side semen evaluation and sperm morphology. Both were used in this project to provide context for two flow cytometric assays used to evaluate sperm samples. As technology advances other assays to test semen quality could be implemented to improve the predictive power of the BBSE, and enable identification of less desirable traits. It is important to acknowledge that it is highly unlikely, given the complexity of the reproductive process, that a single sperm characteristic will reflect the true fertilization potential of a semen sample or the ability of the bull to successfully serve a female. Nevertheless, a combination of diverse laboratory assays, detecting different characteristics of the ejaculate, will more accurately reflect semen fertility than a single test (Kastelic & Thundathil 2008). Identifying a set of semen characteristics considered important for conception, and incorporating these into the BBSE could improve monitoring and/or prediction of semen attributes and bull fertility.

Sperm DNA is a tightly packaged structure, up to 20 times more condensed than the DNA of somatic cells. It is considered inactive with no transcription or translation taking place after final packaging into the mature sperm. The condensation of sperm DNA structure is depending on the substitution of histone with transitional proteins and later on protamines, which bind in the grooves of the DNA, neutralizing the phosphodiester backbone, causing the DNA molecules to coil into tight toroidal structures, making it difficult to access and less susceptible to DNA damage and fragmentation (Balhorn 2007). This condensation enables the production of a small, more hydrodynamic head, characteristic of the sperm, and contributes indirectly, to head shape. Studies in humans have shown that sperm DNA integrity and sperm protamine deficiency and composition, including ratios between different protamines, may results in sub fertility.

Sperm analysis based on flow cytometry methods are constantly evolving and are gradually replacing older, more subjective methods used in assessment of sperm fertilizing capacity (Martinez-Pastor et al. 2010). Specifically, flow cytometry methodologies determining sperm DNA composition or sperm chromatin integrity are emerging as potential predictors of fertility. In brief the sperm chromatin structure assay (SCSA) measures the susceptibility of sperm DNA to denaturation. The assay is based on the metachromatic properties of the acridine orange, and the outcome measures include the DNA Fragmentation Index (DFI) and the High DNA Stainability (HDS). In dairy bulls, SCSA measurements have been shown to be significantly associated with outcome successes after artificial insemination (AI) (Waterhouse *et al.* 2006).

The SCSA has been used to measure DNA integrity of tropically adapted bulls as described in the MLA Report “Markers and genes influencing puberty”. Previous studies (Fortes *et al.* 2012b; D'Occhio *et al.* 2013) have shown that sperm DNA integrity in bulls between 1 and 10 years of age and at 24 months of age correlated with sperm morphology and the percentage of morphological normal sperm cells (PNS). It has also been shown that PNS is significantly correlated with calf output in Australian beef cattle (Holroyd *et al.* 2002) and sperm morphology has been shown to be heritable (Corbet et al. 2013). The SCSA measures parameters which may be associated with other semen quality phenotypes like PNS, however it represents a novel factor not directly reflecting any of the classical sperm quality parameters (Evenson & Jost 2000). The SCSA uses flow cytometry, rather than microscopy, hence it has the potential for high throughput, improved precision and objectivity compared to current microscopic based assessment of semen quality.

The sperm DNA is packaged tightly during spermatogenesis with protamines to ensure safe delivery of the paternal DNA to the oocyte. In the bull and boar there is only one functional form of protamine, P1, which in the bull has been mapped to BTA 29q12-13 (Friedl & Rottmann 1994). In cattle, one study found some variation between bulls in terms of sperm protamine deficiency, using the stain CMA3 and microscopy (Simoes *et al.* 2009). Also sperm protamine deficiency was found in bulls exposed to scrotal insulation (testicular heat insult), associated with the initial drop in semen quality and likely fertility (Rahman *et al.* 2011). A flow cytometry assay for sperm protamine deficiency also based on CMA3 has been developed in humans (Tavalaee *et al.* 2009), and refined further for the use in bulls by our group (Fortes *et al.* 2014). The sperm protamine deficiency assay (SPDA) developed uses snap frozen semen samples and enabled description of variations in terms of protamine deficiency between bulls (Fortes *et al.* 2014). The SPDA could contribute to the interpretation of the SCSA results and further add to the evaluation of sperm DNA packaging. The SCSA and SPDA results were shown to be moderately correlated in the Tropical Composite bull samples that were initially assayed (n=130) (Fortes *et al.* 2014).

It has been proposed that both the SCSA and SPDA could be incorporated into the BBSE and/or semen evaluation in the context of AI. The heritability of the parameters assessed using the SCSA and SPDA is largely unknown as genetic studies have not previously been conducted for sperm chromatin structure traits in any species. Establishing heritabilities of these traits in cattle and the value of performing these tests in the context of BBSE is an important step when implementing new traits and assays. The initial study was carried out on a limited number of Tropical Composite bulls (B.NBP.0604) and is now reported in the literature (Fortes *et al.* 2012b). This research needed to be repeated on a larger number of animals, in particular Brahman bulls, to determine the heritability and usefulness of these parameters in the BBSE.

One part of the current BBSE includes a visual assessment of the semen sample. This includes the evaluation of visual sperm density on a scale from 1-5, where 1 is a gross appearance of clear to slightly turbid and 5 is a creamy appearance. In electroejaculated samples as part of a BBSE the actual sperm concentration is usually not measured, however density is considered to provide a rough estimate of sperm concentration (Beggs *et al.* 2013). In the BBSE manual a scale of sperm density to approximate concentration (sperm per mL) has been provided indicating the relationship. Because epithelia cells and white blood cells may contribute to the change in turbidity a microscopic assessment is needed to demonstrate the presence and absence of these cell types (Beggs *et al.* 2013). In previous work it was found that the visual sperm density measurement collected in the field did not correspond

with the actual measured sperm concentration using a haemocytometer in frozen-thawed samples (unpublished data). The value of the sperm density is questionable and needs further investigation. Furthermore, it is necessary to determine sperm concentration in the majority of newer methodology assessing sperm attributes, this includes any assays based on flow cytometry. More accurate sperm concentration measurements are needed if semen is to be used and diluted for custom freezing or chilling and ensure correct dilution and achieve a minimum sperm number per insemination dose. A haemocytometer or spectrophotometer should be used under these conditions. The measurement and recording of sperm concentration should not be a compulsory part of a BBSE, but a minimum density of 2 (200 x 10^6 sperm/mL) should be used as a threshold when assessing animals for natural mating (Beggs *et al.* 2013). The investigation of the association between visual sperm density measured in the field and the actual sperm concentration measured using a haemocytometer should be further investigated to avoid inconsiderate use of the density parameter, and consider its limitations as a measure of sperm concentration.

The aim of the current project was to evaluate two flow cytometry assays that assess semen quality, particularly sperm chromatin structure: the sperm chromatin structure assay (SCSA) and the sperm protamine deficiency assay (SPDA). The proposal was to compare measurements obtained from two sperm chromatin structure assays with established crush-side semen evaluation, including sperm morphology. These comparisons enabled revision of semen quality evaluation and provided evidence based recommendations to the industry.

The aim of this project was to provide information that can be used to aid the decision making process carried by industry in terms of using or discarding bulls based on their fertility potential and adequacy for breeding.

2. Project objectives

- Conduct a preliminary evaluation of two flow cytometric assays, SPDA and SCSA, and compare these with established crush-side semen evaluation and sperm morphology (Pilot trial – 100 samples).

- Conduct a preliminary evaluation of the relationship between sperm concentration and visual scores of both density and colour (Pilot trial – 100 samples).

- Estimate the heritability of SCSA, SPDA and sperm concentration, and test their value as traits for assisting genetic improvement of herd fertility (1,000 samples; selected to match already genotyped bulls).

- Perform genome wide-association study for these new traits (1,000 samples).

- Re-evaluate crush-side seminal parameters used in BBSE, such as density and motility, in comparison with measurement of semen concentration.

- Provide recommendations about the potential of SCSA and SPDA as future BBSE and Breedplan traits, and the use of visual assessment of density as a measure of sperm concentration.

3. Methodology

A pilot trial was carried with over 100 samples (n = 133 Brahman bulls) which established protocols used in this project (Fortes *et al.* 2014). The bulls in the pilot trial were a sub-group of the animals used in the complete project.

3.1 Animals and samples

In this project data and semen samples from 1,130 2-year-old bulls was used; Brahman (n = 592) and Tropical Composites (n = 538) born from 2004 to 2009 across five properties. Bulls were included on the basis of their sperm sample availability for flow cytometric analyses and availability of genotypes from previous projects. Semen samples from the 2-year-old bulls were collected using electroejculation. Samples of 1 mL undiluted semen were placed in 2 mL eppendorf cryovials and snap frozen in liquid nitrogen, and stored at -80°C until analysis using SCSA and SPDA. The bulls were bred and raised by the Beef CRC and the experimental design as well as the general population description of the CRC has been reported (Burns *et al.* 2013; Corbet *et al.* 2013). Although these bulls were part of previous Beef CRC studies, selection and flow cytometric analyses of the previously collected semen samples described herein constitute new data created specifically to achieve the objectives of this project.

3.2 Bull breeding soundness evaluation and phenotypic data

Bulls of the Beef CRC were subject to measurement of numerous fertility indicator traits. A complete BBSE was attempted when bulls were 12, 18 and 24 months of age. However due to maturity the majority of bulls were able to produce a sperm sample for evaluation at 24 months. Three traits measured as part of the BBSE at 24 months were used in this study: percentage of normal sperm (PNS, %), progressive motility (MOT, %) and density (DEN, visual score 1-5). Details about the collection of samples and measurements of these traits have been described (Burns *et al.* 2013; Corbet *et al.* 2013).

3.3 Sperm concentration in preparation for flow cytometric analysis

Semen samples were snap-frozen in liquid nitrogen and stored as part of the Beef CRC studies. The samples were transported to the School of Veterinary Science, UQ Gatton Campus and stored at -80°C . Immediately prior to flow cytometric analysis the samples were thawed (37°C for 3 min) and sperm concentration determined using the Improved Neubauer chamber and microscopy. A 5 µL aliquot was diluted 1:20 in saline, and 10 µL of the mixture transferred to both sides of the counting chamber with the coverslip fitted. The number of sperm was counted in the 25 squares of the central grid of the chamber.

3.4 Sperm Chromatin Structure Assay (SCSA)

This assay uses the metachromatic properties of acridine orange in the assessment of sperm DNA integrity. Acridine orange fluoresces green when combined with double stranded (intact) DNA and fluoresces red when combined with single stranded (fragmented) DNA. Aliquots of the samples were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, and 1 mM EDTA), to obtain a sperm concentration between 6 and 10 x 10^6 sperm/mL. For each sample, directly after dilution, a total volume of 50 mL was transferred to a Beckman Coulter test tube (Beckman Coulter, Australia Pty, Ltd., Gladesville, NSW). Then, 100 mL acid detergent solution (0.08 M

HCl, 0.15 M NaCl, 0.1% (w/v) Triton X-100, pH 1.2) and after exactly 30 s, 1.2 mL of acridine orange staining solution was added. This solution contained 6 mg AO (acridine orange chromatographically purified, Polysciences, Inc., Warrington, PA, USA) per mL of buffer (0.037 M citric acid, 0.12 M Na_2PO_4, 1.1 mM disodium EDTA, 0.15 M NaCl, pH 6.0). Flow cytometric analysis for SCSA was conducted using flow cytometry; Gallios™ flow cytometer (Beckman Coulter Inc., Miami, FL, USA). The blue excitation laser (488 nm) was used to excite the fluorophore acridine orange and fluorescence was detected on FL1 (525BP40 filter), FL3 (620BP30 filter) and FL4 (675BP20 filter), respectively, at the low flow rate. Every sample was analysed twice; immediately after thawing and determination of concentration, and again within 15 min of thawing. After every six test samples, a reference sample was thawed and analysed to ensure stability of the instrument. Kaluza® software (version 1.1, Beckman Coulter Inc.) was used to analyse the flow cytometric data and generate the three phenotypes for genetic analyses: 1) Percentage of sperm cells with intact chromatin (PIC), 2) DNA fragmentation index (DFI) and 3) percentage of sperm with abnormally high DNA stainability (HDS). SCSA was conducted according to the protocol described by Evenson and Jost (2001). Two DFI values were determined using the FL3 fluorescence (DFI FL3) and the FL4 fluorescence (DFI FL4) for detecting sperm with DNA damage. Likewise, HDS was detected on the two filters: HDS FL3 and HDS FL4. This use of the Gallios cytometer and software, adding an FL4 reading to the standard SCSA was developed previously (Fortes *et al.* 2012b).

3.5 Sperm Protamine Deficiency Assay (SPDA)

The sample was subsequently used for the flow cytometric methodology for SPDA as detailed in our previous work (Fortes *et al.* 2014). Briefly, sperm protamine was assessed using CMA3, a fluorochrome that competes for protamine binding sites at the minor groove of the DNA strand (Evenson *et al.* 1986; Tavalaee *et al.* 2010). Aliquots of the samples were diluted to approx. 50 x 10^6 sperm/mL in Dulbecco's PBS (DPBS, Ca2+ and Mg2+ free), and subsequently washed by centrifugation (500 g for 5 min for all centrifugation washes). The pellet was resuspended into DPBS, split into two aliquots and incubated for 15 min at 37°C in the treatments: 1) no treatment and 2) pooled 6 consecutive samples positive control treated with 5 mM dithiothreitol (DTT). For the pilot trial we performed the positive control for all samples. For the subsequent 1000 samples a pooled sample, for every 6 samples was used as positive control. The samples were washed twice by centrifugation, resuspended into DPBS and washed again by centrifugation. The samples were then resuspended in 0.25 mg/mL CMA3 in McIlvaine's buffer (17 mM citric acid, 164 mM Na_2HPO_4 and 10 mM $MgCl_{26}H_2O$; pH 7.0) and incubated at room temperature for 1 h in the dark. Samples were then washed three times by centrifugation and the concentration was adjusted to 5–10 x 10^6 sperm/mL for flow cytometric analysis (Gallios™ flow cytometer; Beckman Coulter Inc., Miami, FL, USA). Two excitation solid state lasers, violet laser (405 nm) and blue laser (488 nm), were used and fluorescence was detected on FL9 (450BP50 filter), FL10 (550BP40 filter) and FL2 (575BP30 filter), respectively, at the low flow rate. Kaluza® software (version 1.1, Beckman Coulter Inc.) was used to analyse the flow cytometric data and generate the three phenotypes for genetic analyses: the proportion of sperm cells in each sample with high, medium and low CMA3 binding (HCB, MCB and LCB). The degree of CMA3 binding is an indirect measurement of protamine content.Sperm with HCB are considered deprotaminated, while sperm with MCB are considered to have protamine deficiency and sperm with LCB are likely to have normal levels of protamine content (or protamine intact).

3.6 Genotypes: genome-wide SNP panel

Genomic DNA was extracted from blood samples for genotyping. SNP chips with genetic markers distributed across the genome were used; these are commercialised by Geneseek® and Illumina (Matukumalli *et al.* 2009). Both chips use the Illumina chemistry and platform for genotyping. Genotyping services were provided by Animal Genetics Laboratory (AGL), at the University of Queensland, Gatton. Genotype calling and quality control were performed using Genome Studio (Illumina, 2015). Genotyping methods and quality control were described by Fortes et al, Lyons et al and de Camargo et al (Fortes *et al.* 2012a; Fortes *et al.* 2013; Lyons *et al.* 2014; de Camargo *et al.* 2015). Genotype data from all previous studies were merged and imputation with Beagle software was performed (Browning & Browning 2009) to form the genotype dataset for the bulls evaluated in this project. As a consequence, genotypes for 68,406 single nucleotide polymorphisms (SNP) were available for 1,130 bulls with the new phenotypes.

3.7 Genome-wide association studies (GWAS)

The association of each SNP with each phenotype was examined for genotyped animals using a mixed model analysis of variance with SVS software (Golden Helix Inc). The mixed model included fixed effects, random additive polygenic effects of animal (pedigree relationship matrix) and the observed animal genotype for the SNP (coded as 0, 1 or 2 to represent the number of copies of the B allele) and a random residual effect. The same fixed effects included contemporary group (animals born in the same year and raised together, also termed “cohort”) and age was fitted as a covariant in the model. For SCSA results, “day of assay” was included as a fixed effect in the model as it significantly affected the measurements of PIC and HDS, which are more dependent of the intensity of the laser on the day. Analyses were conducted within breed. Brahman bulls were analysed separately from Tropical Composite bulls, because across breed GWAS occasionally give false negative results. The quantitative trait loci (QTL) results, seen as genomic regions of high SNP association in GWAS, sometimes disappear when two different breeds are analysed together.

Genotypes used in GWAS were also used to estimate genomic heritabilities and pair-wise correlations between studied traits. The heritabilities presented in the results section were based on genomic data; because a genomic relationship matrix was used to ascertain similarity between individuals. The Qxpak5 software (Perez-Enciso & Misztal 2011) was used to generate heritabilities and genomic correlations. Univariate models estimated heritabilities and bivariate models estimated genomic correlations.

4. Results

A pilot trial was conducted with over 100 samples (n = 133 Brahman bulls) and initial results were published (Fortes *et al.* 2014). The pilot trial accomplished the first two project objectives by 1) conducting preliminary comparisons between SPDA, SCSA, crush-side semen evaluation and sperm morphology; and 2) evaluation of the relationship between sperm concentration and visual scores of both density and colour. The pilot trial was successful in establishing the SPDA protocol. Significant phenotypic correlations between SCSA, SPDA and some traditional semen quality parameters (i.e. PNS, sperm motility and concentration) were reported. Further, the imprecision associated with visual scores of density and colour, which were not

significantly correlated with sperm concentration, were demonstrated. The estimated correlations with sperm concentration were 0.10±0.09 for density and 0.07±0.09 for colour (neither significant). The establishment of protocols and significant correlations between SCSA and PNS justified the continuation of this research.

Results for the complete project (n = 1,130 bulls) are presented in two sub-sections. The first sub-section gives a brief description of studied fertility indicators and summarizes the data by presenting means and standard deviations of each studied trait (phenotype) per breed. Estimated phenotypic and genetic correlations as well as heritabilities were reported. The second sub-section details the results found in the GWAS, and are presented per breed.

4.1 Fertility indicators and their correlations

A brief description of the fertility indicator traits measured followed by descriptive statistics is given in Tables 1 and 2. Actual age at semen collection (Age) for bull breeding soundness evaluation (BBSE) is also provided in Table 1 as this was used as a fixed effect in the GWAS models. All samples and measurements were taken at approximately 24 months, when most bulls have reached puberty and are young adults.

Table 1. Brief description of all semen traits, mean and standard deviation (SD) for Brahman and Tropical Composite (TC) bulls. Based on electroejaculated samples collected at 24 months of age.

		Brahman		TC	
Traits	**Description**	**Mean**	**SD**	**Mean**	**SD**
Age	Age at semen collection for BBSE, days	707.55	23.61	725.49	24.24
DEN	Sperm density visual score, 1-5	3.23	0.79	3.48	0.60
CON	Sperm concentration in sample, $x10^6$ sperm/mL	266.11	287.41	237.18	238.23
MOT	Progressive sperm motility, %	74.58	19.96	76.09	20.76
PNS	Percentage of morphologically normal sperm, %	71.24	21.32	72.46	18.72
SPDA	**Sperm protamine deficiency assay**				
LCB	Low CMA3 binding, % (intact protamine)	84.50	14.78	87.97	11.27
MCB	Medium CMA3 binding, % (protamine deficient)	14.86	14.42	12.00	11.25
HCB	High CMA3 binding, % (deprotaminated)	5.99	10.68	5.44	6.06
SCSA	**Sperm chromatin structure assay**				
PIC3	Sperm with intact chromatin on FL3, %	89.85	8.71	89.27	6.89
PIC4	Sperm with intact chromatin on FL4, %	86.95	10.26	85.32	11.28
DFI3	DNA fragmentation index on FL3, %	3.77	4.23	4.47	4.73
DFI4	DNA fragmentation index on FL4, %	6.41	7.13	8.38	10.38
HDS3	High DNA stainability on FL3, %	6.38	6.76	6.27	3.96
HDS4	High DNA stainability on FL4, %	6.64	7.23	6.29	3.94

Phenotypic correlations were estimated pair-wise to re-assess initial results from the pilot trial (Fortes *et al.* 2014). Initial results were based on 113 Brahman bulls, a small sub-group of the 1,130 bulls analysed. Heritabilities and genomic correlations were also estimated within breed for Brahmans and Tropical Composites, respectively (Tables 2 and 3).

Table 2. Semen traits in Brahman (n = 592): phenotypic correlations (below diagonal), heritabilities (diagonal) and genomic correlations (above diagonal).

	PIC3	DFI3	HDS3	PIC4	DFI4	HDS4	LCB	MCB	HCB	CON	DEN	PNS	MOT
PIC3	0.25	-0.83	-0.88	0.89	-0.64	-0.86	0.18	-0.18	-0.34	-0.06	-0.23	0.04	0.27
DFI3	-0.65	0.43	0.40	-0.83	0.80	0.37	-0.34	0.33	0.50	-0.04	0.10	-0.20	-0.32
HDS3	-0.88	0.22	0.22	-0.71	0.19	0.99	-0.05	-0.02	0.08	0.11	0.37	0.14	-0.14
PIC4	0.91	-0.71	-0.73	0.28	-0.90	-0.70	0.38	-0.37	-0.36	-0.01	-0.09	0.19	0.38
DFI4	-0.43	0.83	0.039	-0.71	0.29	0.17	-0.66	0.44	0.47	-0.08	-0.16	-0.44	-0.42
HDS4	-0.87	0.19	0.997	-0.72	0.02	0.22	-0.04	-0.04	0.05	0.11	0.39	0.16	-0.12
LCB	0.20	-0.31	-0.06	0.30	-0.37	-0.05	0.48	-0.98	-0.74	-0.04	0.02	0.23	0.24
MCB	-0.16	0.30	0.02	-0.26	0.37	0.01	-0.98	0.47	0.82	0.06	0.02	-0.22	-0.25
HCB	-0.14	0.31	-0.02	-0.20	0.32	-0.03	-0.75	0.73	0.30	-0.00	0.07	-0.10	-0.20
CON	-0.03	-0.04	0.067	0.00	-0.07	0.06	-0.05	0.078	0.028	0.37	0.44	0.08	0.02
DEN	-0.09	-0.03	0.14	-0.04	-0.09	0.14	0.06	-0.03	-0.04	0.34	0.21	0.29	0.31
PNS	0.086	-0.22	0.028	0.21	-0.33	0.03	0.25	-0.25	-0.20	0.08	0.09	0.42	0.40
MOT	0.18	-0.30	-0.05	0.24	-0.31	-0.03	0.21	-0.22	-0.19	0.00	0.30	0.21	0.19

Table 3. Semen traits in Tropical Composite (n = 538): phenotypic correlations (below diagonal), heritabilities (diagonal) and genomic correlations (above diagonal).

	PIC3	DFI3	HDS3	PIC4	DFI4	HDS4	LCB	MCB	HCB	CON	DEN	PNS	MOT
PIC3	0.42	-0.83	-0.75	0.78	-0.56	-0.74	0.44	-0.44	-0.41	0.05	0.04	0.30	0.27
DFI3	-0.83	0.41	0.26	-0.79	0.78	0.25	-0.48	0.48	0.45	-0.20	-0.11	-0.36	-0.33
HDS3	-0.75	0.25	0.43	-0.40	0.06	1.00	-0.19	0.19	0.18	0.15	0.08	-0.10	-0.17
PIC4	0.77	-0.79	-0.40	0.41	-0.94	-0.40	0.54	-0.54	-0.47	0.11	0.11	0.33	0.28
DFI4	-0.56	0.76	0.06	-0.94	0.41	0.05	-0.51	0.51	0.45	-0.17	-0.14	-0.32	-0.24
HDS4	-0.74	0.24	1.00	-0.39	0.05	0.44	-0.19	0.19	0.18	0.15	0.08	-0.11	-0.18
LCB	0.45	-0.49	-0.20	0.54	-0.51	-0.20	0.39	-0.99	-0.81	0.17	0.07	0.35	0.31
MCB	-0.45	0.49	0.20	-0.53	0.51	0.20	-1.00	0.39	0.81	-0.17	-0.07	-0.34	-0.31
HCB	-0.42	0.45	0.18	-0.47	0.44	0.18	-0.81	0.81	0.38	-0.23	-0.09	-0.26	-0.31
CON	0.05	-0.20	0.16	0.10	-0.17	0.16	0.18	-0.18	-0.23	0.36	0.37	0.04	0.13
DEN	-0.01	-0.11	0.14	0.09	-0.15	0.13	0.11	-0.11	-0.11	0.48	0.19	0.30	0.51
PNS	0.31	-0.36	-0.10	0.33	-0.31	-0.11	0.35	-0.35	-0.28	0.04	0.01	0.40	0.60
MOT	0.27	-0.25	-0.17	0.29	-0.25	-0.17	0.33	-0.32	-0.32	0.14	0.23	0.36	0.20

The correlation between density and concentration is of practical interest, since density visual scores are often used as an approximation (or even to substitute) sperm concentration in semen evaluation. We found low to moderate correlations between density scores and sperm concentration (see Tables 2 and 3). To further illustrate the relationship and the nature of these correlations we provide scatter plots (Figure 1).

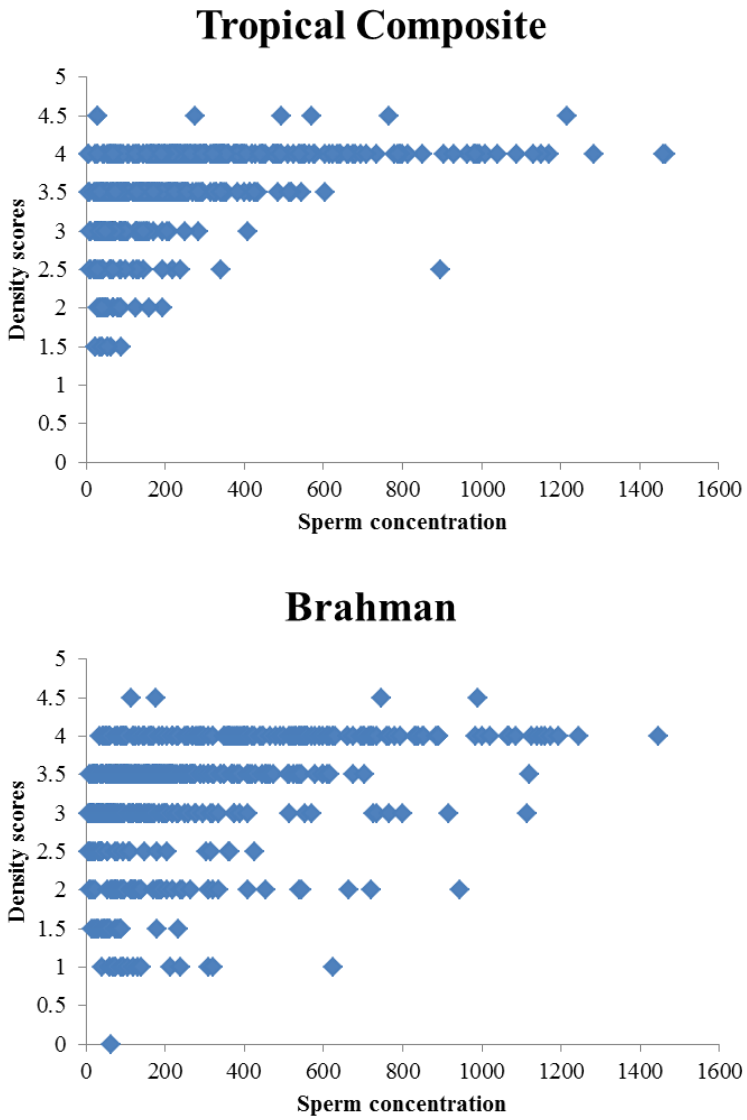

Figure 1. Scatter plot of the correlation between visual density scores (1-5) and sperm concentration (x10^6 sperm/mL) in Tropical Composite (n = 538) and Brahman bulls (n = 592). Note that 44 Brahman samples and 52 Tropical Composite samples had very low sperm concentration (<200 x10^6 sperm/mL) and at the same time scored high (>4) in the visual assessment.

4.2 GWAS results

These GWAS represent the first report on genetic architecture of sperm concentration and sperm chromatin traits (SCSA and SPDA measurements). We found some evidence for genes of major effect, supported by genome-wide significant SNP associations ($P < 10^{-6}$). For example, PNS and LCB (intact protamine) had significant SNP associations concentrated in X chromosome regions. Other phenotypes though, like HDS observed with SCSA seemed to hold true to complex traits expectations: no major gene or QTL, instead presence of medium to low SNP associations distributed across the genome. Overall, we observe that chromosome X holds important genetic information for many phenotypes related to male fertility. Trait and breed specific results are reported below and summarized in Table 4.

For visualization of the major QTL findings, each GWAS is also presented as a Manhattan plot (Figures 2-9; *y*-axis $-\log_{10}$ *P*-values and *x*-axis chromosomal positions as per UMD3.1 bovine reference genome). Note the differences in the *y*-axis between figures, which were necessary to accommodate variations in terms of SNP significance for the studied trait.

Table 4. Summary of genome-wide association studies for SCSA, SPDA and concentration; Number of single nucleotide polymorphisms (SNP) associated at 5 *P*-value thresholds* (related false discovery rates) and number of SNP that explained 1-3 or more of the additive genetic variance.

Fertility	SCSA FL3			SCSA FL4			SPDA			CON
Indicators	PIC	DFI	HDS	PIC	DFI	HDS	LCB	MCB	HCB	
Brahman										
$P < 5x10^{-2}$	3699 (0.92)	3762 (0.91)	3586 (0.95)	4250 (0.80)	4432 (0.77)	3556 (0.96)	4540 (0.75)	4407 (0.77)	3672 (0.93)	3613 (0.94)
$P < 1x10^{-2}$	803 (0.85)	731 (0.93)	772 (0.88)	1015 (0.67)	1381 (0.49)	760 (0.90)	1367 (0.50)	1322 (0.52)	807 (0.85)	785 (0.87)
$P < 1x10^{-3}$	92 (0.74)	105 (0.65)	79 (0.86)	162 (0.42)	300 (0.23)	80 (0.85)	499 (0.14)	510 (0.13)	112 (0.61)	178 (0.38)
$P < 1x10^{-4}$	17 (0.40)	28 (0.24)	11 (0.62)	41 (0.17)	136 (0.05)	13 (0.53)	173 (0.04)	184 (0.04)	42 (0.16)	99 (0.07)
$P < 1x10^{-5}$	6 (0.11)	5 (0.14)	1 (0.68)	12 (0.06)	92 (0.01)	1 (0.68)	60 (0.01)	59 (0.01)	17 (0.04)	16 (0.04)
$P < 1x10^{-6}$	**2 (0.03)**	**3 (0.02)**	0	**2 (0.03)**	**47 (0.00)**	0	**33 (0.00)**	**30 (0.00)**	**5 (0.01)**	**1 (0.07)**
%Var > 1	1299	1193	1245	1565	1873	1236	1896	1794	1214	1150
%Var > 2	71	84	62	137	259	62	420	452	85	148
%Var > 3	7	9	5	18	107	6	110	89	36	62
Tropical Composite										
$P < 5x10^{-2}$	3935 (0.87)	4664 (0.73)	3793 (0.90)	3841 (0.89)	4317 (0.79)	3820 (0.89)	3513 (0.97)	3481 (0.98)	3301 (1.03)	4273 (0.80)
$P < 1x10^{-2}$	990 (0.69)	1808 (0.38)	741 (0.92)	772 (0.88)	1216 (0.56)	744 (0.92)	744 (0.92)	745 (0.92)	678 (1.00)	1214 (0.56)
$P < 1x10^{-3}$	92 (0.74)	818 (0.08)	89 (0.77)	82 (0.83)	178 (0.38)	86 (0.79)	86 (0.79)	83 (0.82)	86 (0.79)	160 (0.43)
$P < 1x10^{-4}$	10 (0.68)	440 (0.02)	10 (0.68)	2 (3.41)	12 (0.57)	11 (0.62)	7 (0.98)	7 (0.98)	10 (0.68)	8 (0.85)
$P < 1x10^{-5}$	2 (0.34)	80 (0.01)	0	1 (0.68)	0	0	1 (0.68)	1 (0.68)	1 (0.68)	0
$P < 1x10^{-6}$	0	**14 (0.00)**	0	0	0	0	**1 (0.07)**	**1 (0.07)**	0	0
%Var > 1	2120	3040	1955	1982	2518	2012	1618	1584	1454	2158
%Var > 2	158	903	131	121	250	134	97	96	94	178
%Var > 3	10	439	9	2	12	10	4	3	5	2

*P-values lower than $1x10^{-6}$ are represented in bold; these are normally considered genome-wide significant.

The associations found in the Brahman population for the SCSA phenotypes were visualized as Manhattan plots (Figures 2, 3 and 4). We found evidence of associated SNP, candidate genes (significant QTL) in chromosomes 11 and X for DFI. Chromosomes 9 and 11 presented SNP associated with the PIC3 and PIC4. For PIC4, chromosome X also had significant associations. No major QTLs could be identified for HDS in Brahman.

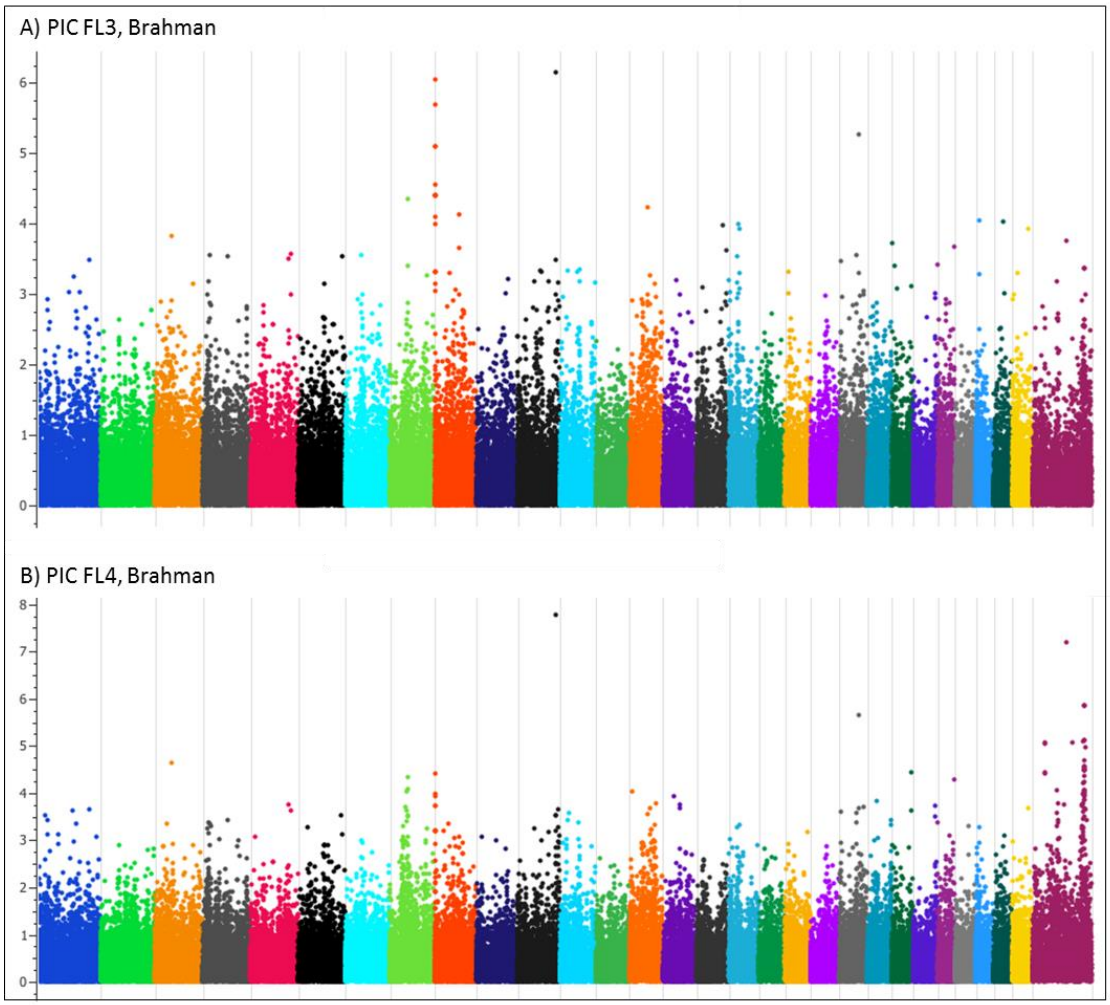

Figure 2. Manhattan plot: genome-wide association results in Brahman bulls for percentage of intact sperm cells (PIC) observed with FL3 (A) and FL4 (B) in the sperm chromatin structure assay (SCSA). In the *y*-axis are $-\log_{10}$ *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 bovine reference genome).

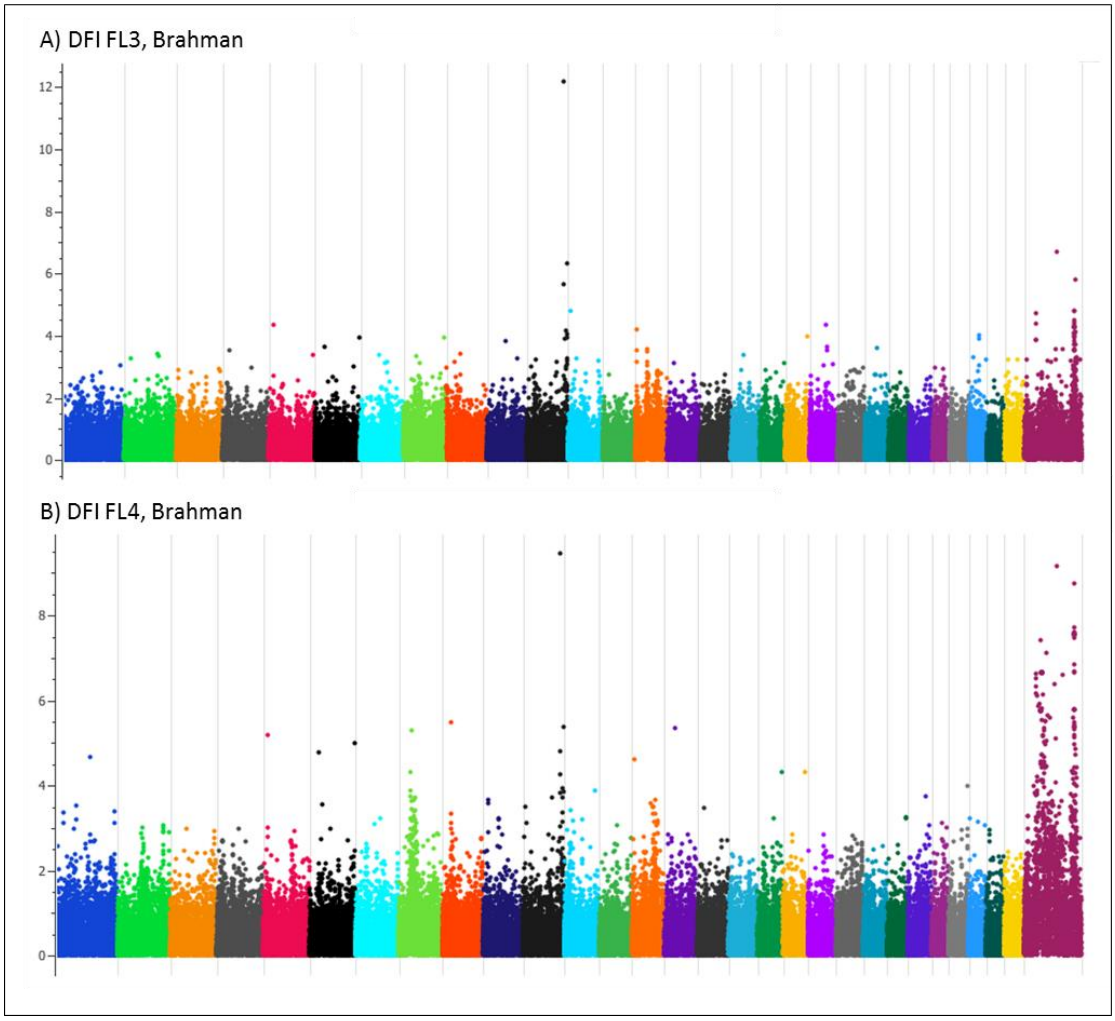

Figure 3. Manhattan plot: genome-wide association results for DNA Fragmentation Index (DFI), measured as percentage of sperm cells in the sperm chromatin structure assay (SCSA). A) Brahman results for DFI observed in FL3. B) Brahman results for DFI observed in FL4. In the *y*-axis are $-\log_{10}$ *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 bovine reference genome). Each colour represents a chromosome from 1 to X (left to right). Note significant results in chromosomes 11 and X (data points above 6).

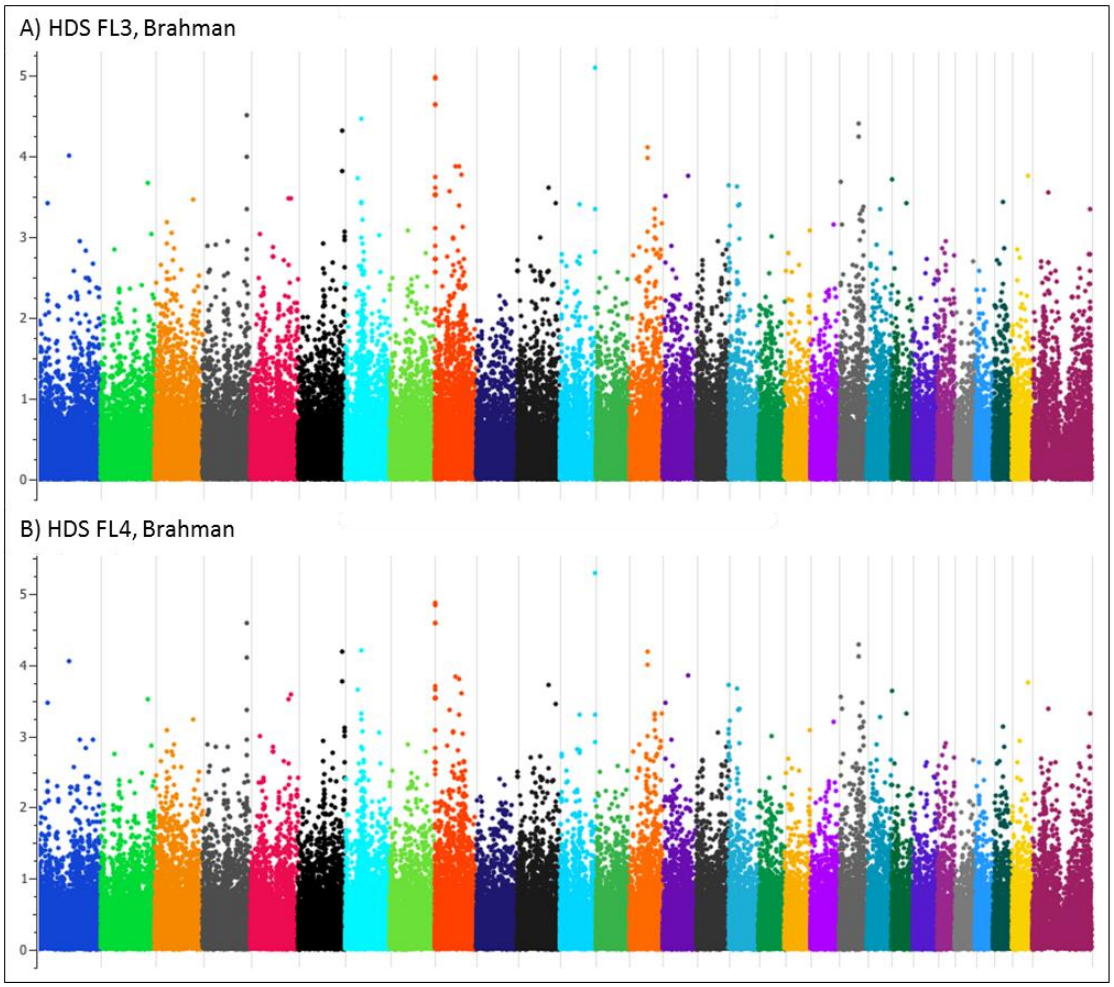

Figure 4. Manhattan plot: genome-wide association results for percentage of HDS observed with FL3 and FL4 in the sperm chromatin structure assay (SCSA) in Brahman bulls. In the *y*-axis are $-\log_{10}$ *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 bovine reference genome). Each colour represents a chromosome from 1 to X (left to right).

The associations found in the Tropical Composite population for the SCSA phenotypes were visualized as Manhattan plots (Figures 5, 6 and 7). We found evidence for significant SNP associations in chromosome X for DFI. For PIC4, chromosome 1 also had a SNP approaching genome-wide significance. Chromosome X had the majority of the SNP above the $P < 0.001$ threshold for both PIC3 and PIC4. Similarly to what we observed for Brahmans, no major QTL could be identified for HDS in Tropical Composites.

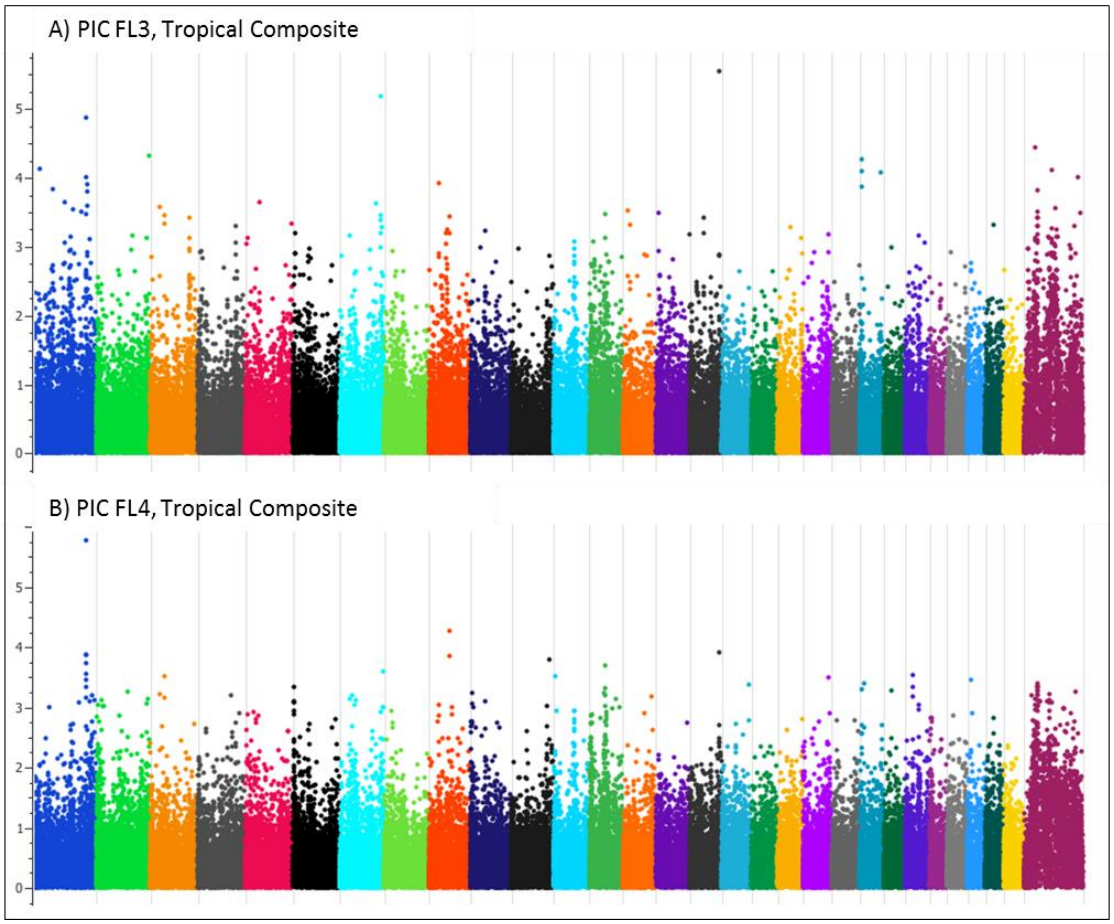

Figure 5. Manhattan plot: genome-wide association results for the percentage of sperm cells with intact chromatin (PIC) observed with FL3 and FL4 in the sperm chromatin structure assay (SCSA) in Tropical Composite bulls. In the *y*-axis are $-\log_{10}$ *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 bovine reference genome). Each colour represents a chromosome from 1 to X (left to right).

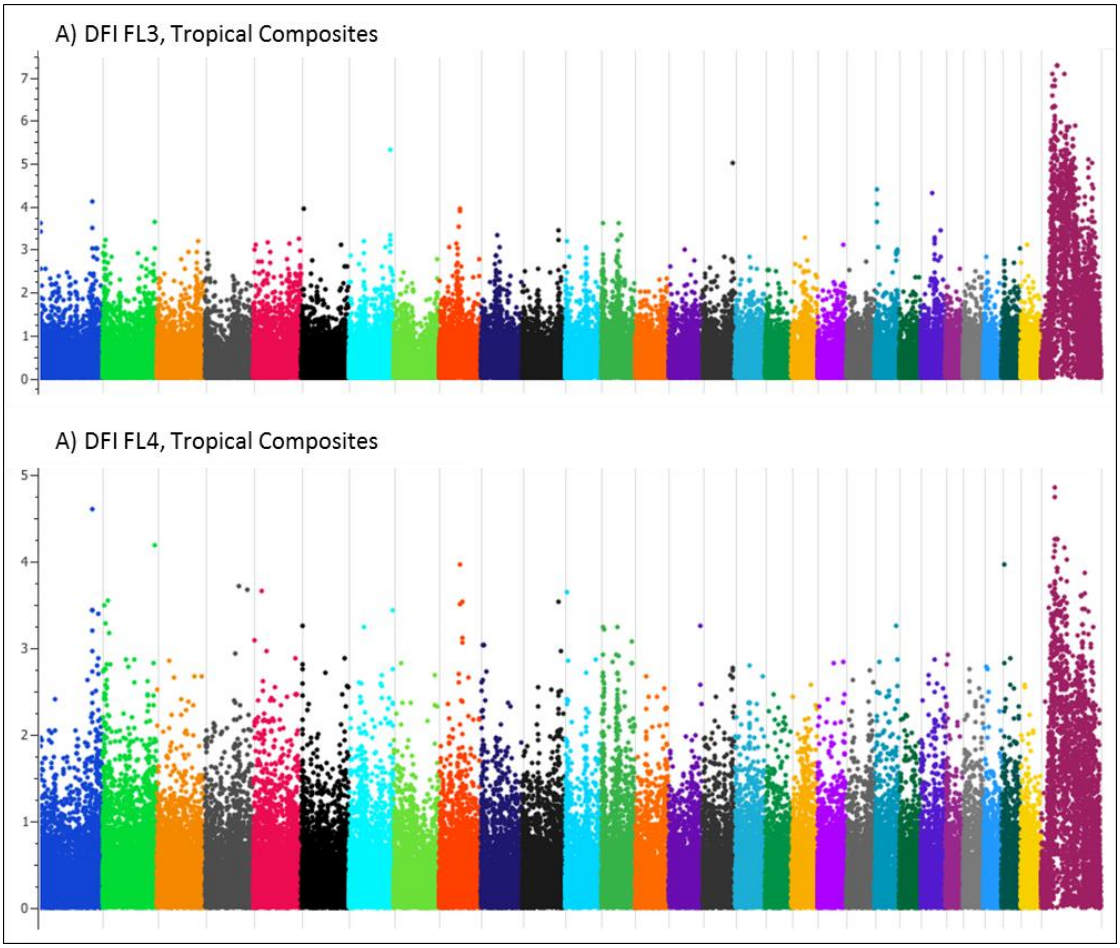

Figure 6. Manhattan plot: genome-wide association results for the DNA Fragmentation Index (DFI) observed with FL3 and FL4 in the sperm chromatin structure assay (SCSA) in Tropical Composite bulls. In the *y*-axis are $-\log_{10}$ *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 bovine reference genome). Each colour represents a chromosome from 1 to X (left to right).

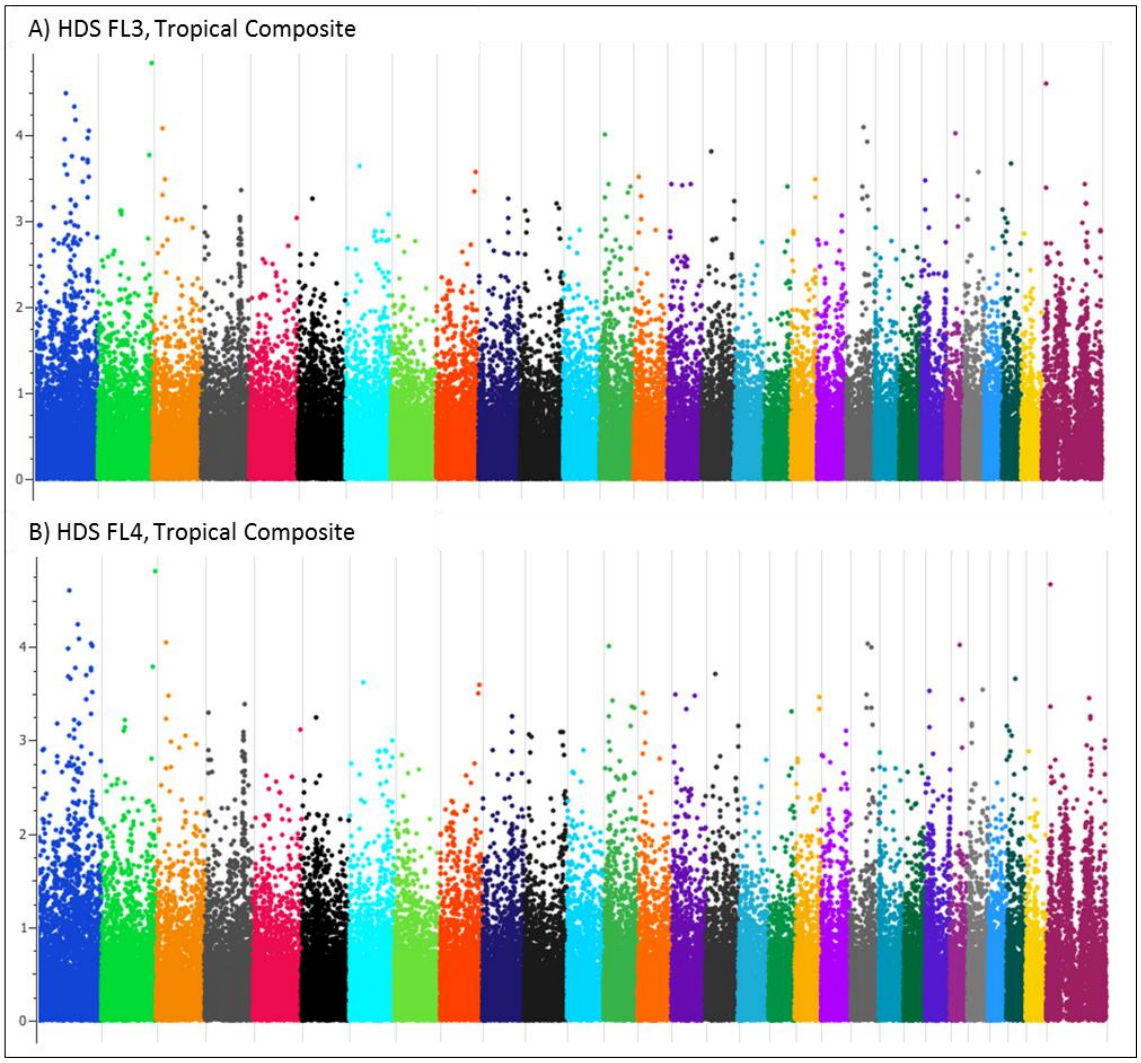

Figure 7. Manhattan plot: genome-wide association results for percentage of HDS observed with FL3 and FL4 in the sperm chromatin structure assay (SCSA) in Tropical Composite bulls. In the *y*-axis are $-\log_{10}$ *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 bovine reference genome). Each colour represents a chromosome from 1 to X (left to right).

Results for GWAS carried with the SPDA phenotypes are presented as Manhattan plots for Brahman (Figure 8) and Tropical Composites (Figure 9). All of the genome-wide significant SNP for HCB and MCB discovered for Brahman and Tropical Composites were mapped to chromosome X. This QTL is more evident in the Brahman results. These results indicated that genes located on chromosome X could play a role in determining the levels of protamine content observed in bovine sperm.

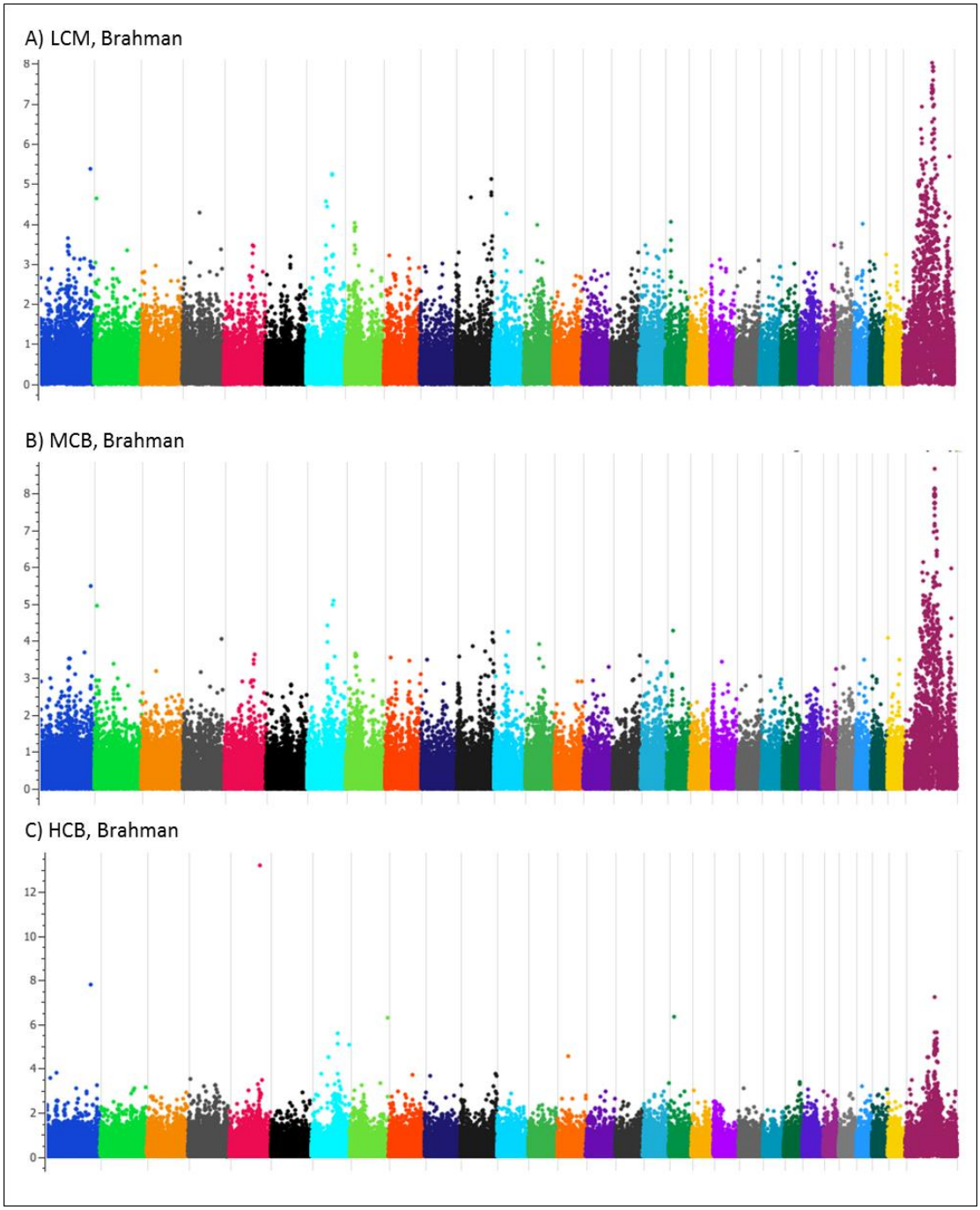

Figure 8. Manhattan plot for Brahman bulls: genome-wide association results for percentage of sperm cells with low, medium and high CMA3 binging (LCB, MCB and HCB) observed with the sperm protamine deficiency assay (SPDA). CMA3 binding is an indirect measurement to estimate levels of protamine content; low CMA3 binging corresponds to expected levels of protamine (normal), while high CMA3 binging corresponds to low levels of protamine. In the *y*-axis are $-\log_{10}$ *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 bovine reference genome). Each colour represents a chromosome from 1 to X (left to right).

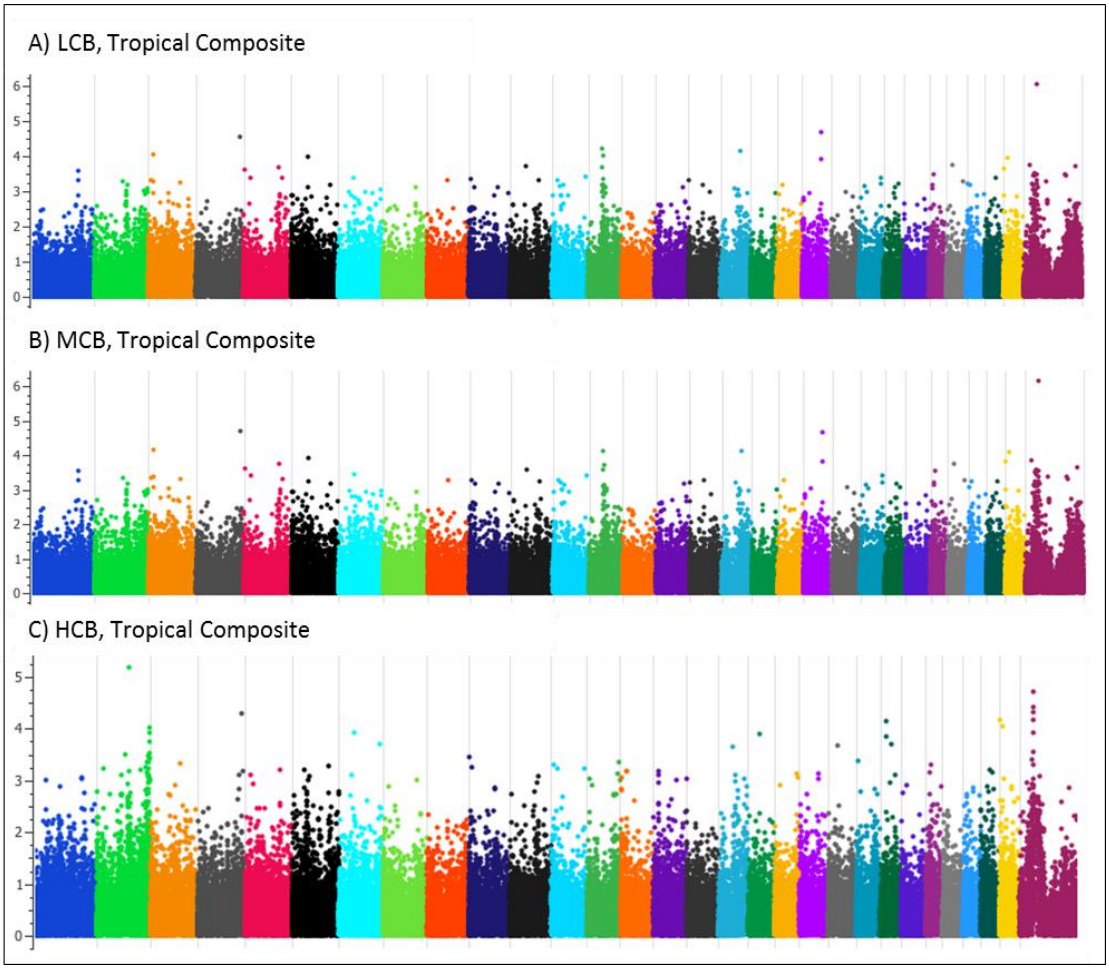

Figure 9. Manhattan plot for Tropical Composite bulls: genome-wide association results for percentage of sperm cells with low, medium and high CMA3 binging (LCB, MCB and HCB) observed with the sperm protamine deficiency assay (SPDA). CMA3 binding is an indirect measurement to estimate levels of protamine content; low CMA3 binging corresponds to expected levels of protamine (normal), while high CMA3 binging corresponds to low levels of protamine. In the *y*-axis are $-\log_{10}$ *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 bovine reference genome). Each colour represents a chromosome from 1 to X (left to right).

5. Discussion

5.1 Sperm Protamine Deficiency Assay (SPDA)

In the initial pilot study a method to measure sperm protamine deficiency (SPDA) in cattle (Fortes *et al.* 2014) was established. This method, termed SPDA has now been applied to a total of 1,130 bulls of two breeds: Brahman and Tropical Composite. The heritabilities estimated for LCB, MCB and HCB ranged from 0.30 to 0.47 in Brahman and were close to 0.38 in Tropical Composites. Heritabilities in these ranges are higher than the majority reported for fertility traits, specially female traits (Cammack *et al.* 2009). Further, they are in the range of heritabilities expected for scrotal circumference, a trait known to respond well to selection in breeding programs (Amyes & Morris 2009). The results indicate that protamine deficiency is correlated with sperm chromatin susceptibility to fragmentation. Also sperm protamine deficiency was correlated with PNS and MOT (genomic correlations

ranging from 0.22 to 0.35). The phenotypic correlations noted between deficient levels of protamine and higher DNA susceptibility to fragmentation confirmed the initial results (Fortes *et al.* 2014). These correlations between SPDA observations and bull fertility indicators, suggest that protamine levels contribute to bull fertility.

5.2 Sperm Chromatin Structure Assay (SCSA)

Sperm DNA susceptibility to fragmentation, measured as DFI in the SCSA, has been associated to fertility indicators and field fertility in a number of studies and species (Evenson 2006; Waterhouse *et al.* 2006; Boe-Hansen *et al.* 2008; Waterhouse *et al.* 2009; D'Occhio *et al.* 2013). In dairy cattle, the utility of SCSA as a predictor of artificial insemination outcomes was demonstrated (Waterhouse *et al.* 2006). In this project, it was confirmed that DFI is moderately correlated with PNS. The genetic correlations between DFI3, DFI4 and PNS were estimated as -0.20 and -0.43 in Brahman bulls. In Tropical Composites bulls, correlations between DFI3, DFI4 and PNS were -0.32 and -0.36, respectively. These results indicate that higher levels of sperm abnormalities are correlated with higher levels of sperm DNA fragmentation. The heritability of DFI3 and DFI4 was estimated to range between 0.29 and 0.43 (both breeds). Similarly to above discussion about SPDA heritability, heritability estimative suggests that DFI would respond to selective breeding. In summary, sperm DNA fragmentation is a heritable trait expected to influence fertility in Brahman and Tropical Composite bulls. This expectation needs to be confirmed in fertility trials.

In humans, any more than 30% of sperm chromatin damage is considered a real impediment for male fertility (Evenson et al 1999; Evenson et al 2013). In cattle, a threshold such as this has never been established. To establish this threshold in tropical cattle and improve our understanding of the prevalence of DNA fragmentation issues in Australia fertility trials are necessary. Current knowledge of prevalence in Australian bulls has used the human threshold to define the problem, reporting that 513 bulls (91%) had <15 %DFI, 24 bulls (4%) had 15 to 27 %DFI, and 28 bulls (5%) had >27 %DFI (D'Occhio *et al.* 2013). Out of the 592 Brahman bulls studied in this project 574 (96%) had <15 %DFI, 8 (1%) had 15 to 27 %DFI and 3 (0.5%) had >27 %DFI; using the DFI3 measurement. Similar results were observed for Tropical Composites: 491(96%) had <15 %DFI, 17 (3%) had 15 to 27 %DFI and 2 (0.4%) had >27 %DFI. The numbers of bulls with %DFI higher than 15 or 17 were a little higher when DFI4 was counted (data not shown). Importantly, both Brahman and Tropical Composite bulls studied in this project were from the Beef CRC project. This is therefore a bias cohort of bulls, raised under certain conditions that may not represent the Australian prevalence numbers for sperm DNA fragmentation.

5.3 Sperm concentration and density

The correlations between sperm concentration (sperm per mL) measured using an improved Neubauer haemacytometer with microscopy and the visual assessments of density and colour were not significant. Given the lack of significant ($P > 0.05$) correlations, visual assessment is not an acceptable substitute for sperm concentration in the evaluation of bull fertility in the field. A complete BBSE should always include an objective measurement of sperm concentration (i.e. number of sperm cells per mL of ejaculate). Diluting semen for artificial insemination based on density measure is a field practice sometimes used in the absence of actual concentration counts. This practice should be strongly discouraged as it could lead to poor quality semen, more specifically semen straws with lower then recommended concentration, and suboptimal dilution; semen extender to sperm ratio, and therefore lower conception rates . Furthermore, including sperm concentration in the standard

BBSE will enable easier implementation of more advanced technologies, such as flow cytometric assays. Measuring a variety of sperm attributes either in the field or at a central laboratory is likely to improve the capacity to predict bull fertility. The visual sperm density parameter should be considered as an insufficient substitute for sperm concentration in electroejculated semen samples.

5.4 Genome-wide association study

Polymorphisms mapped to the X chromosome were associated to scrotal circumference, percentage of normal sperm and inhibin levels as a result of previous GWAS studies (Fortes *et al.* 2012a; Fortes *et al.* 2013). Herein, we confirmed the importance of X for male fertility by discovering SNP associations in this chromosome for sperm chromatin phenotypes measured with SCSA and SPDA. Further investigation into the role of genes within these QTLs on chromosome X would help to dissect these results and pinpoint causative mutations for sperm chromatin phenotypes. The discovery of causative mutations could be one avenue to incorporate selection for lower DFI and optimal protamine binding in tropically adapted bulls, improving overall fertility.

6. Conclusion

Sperm chromatin phenotypes, such as susceptibility to fragmentation and levels of protamine deficiency are heritable traits, which are genetically correlated with other indicators of bull fertility, such as percentage of morphologically normal sperm. Polymorphisms mapped to the X chromosome were associated with a number of fertility indicators in bulls, herein and as a result of previous studies (Fortes *et al.* 2012a; Fortes *et al.* 2013). Further investigation into the role of chromosome X genes in the context of bull fertility would help to dissect these results and pinpoint functional mutations for sperm chromatin phenotypes. Functional mutations can aid genomic selection. Dissecting these results will also improve our understanding of bull fertility and may deliver new management strategies.

7. Recommendations and future directions

7.1 Visual density scores should not substitute sperm concentration.

In a complete BBSE, a visual density score cannot replace a measure of sperm concentration. The use of visual density scores as an approximation to concentration in any context, and especially when performing semen dilution for AI should be strongly discouraged. The risk of dilution error is high particularly as many samples scored high on density ($\geq$4) however were found to have low concentration (<200 x 10^6 sperm /mL). Dilution errors in field may lead to poor quality of AI doses and as a consequence poor pregnancy outcomes. Assessment of concentration of a semen sample is likely to be important in the future implementation of objective measures of any sperm attributes, as concentration or number of sperm can affect measurement of other semen attributes.

7.2 Fertility trials for SCSA and SPDA

The correlations between sperm chromatin traits (measurements of SCSA and SPDA) with traditional fertility indicators, such as PNS and MOT should encourage further research. We recommend initial further investigation of the SPDA measures

and association directly with fertility using *in vitro* using in vitro fertilization (IVF) and intracytoplasmatic sperm injection (ICSI), with representative bull semen samples with low or high levels of the three categories of CMA3 binding; LCB, MCB and HCB. This should establish the direct association between protamine deficiency and fertility. The studies should include the SCSA as well; however the novelty is the SPDA. If a correlation is found based on *in vitro* fertility results, further field trial and development of field friendly methodologies should be encouraged.

7.3 SCSA and SPDA will respond to genetic improvement strategies

We have estimated moderate heritability for a variety of phenotypes measured by SCSA and SPDA. Therefore, traditional breeding strategies employed in animal selection would be effective in terms of improving herd averages in terms of chromatin integrity (lower DFI) and protamination (lower protamine deficiency). In practice, objective measurements such as SCSA, SPDA, PNS and SC will respond to genetic improvement strategies better than visual assessments of motility and mass activity (this recommendation is based on the estimated heritabilities).

7.4 Use of genetic markers can help selection for bull fertility

SCSA and SPDA results will help to identify mutations in chromosome X that are highly significant for overall bull fertility.

8. Related publications

The research carried as part of this project is related to publications listed below, all supported by MLA funding. Further, related research was also selected for presentation at to scientific conferences: AAABG 2015 and ICAR 2016.

- de Camargo G.M.F., Porto-Neto L.R., Kelly M.J., Bunch R.J., McWilliam S.M., Tonhati H., Lehnert S.A., Fortes M.R.S. & Moore S.S. (2015) Non-synonymous mutations mapped to chromosome X associated with andrological and growth traits in beef cattle. BMC Genomics 16, 384.
- Fortes M.R.S., Satake N., Corbet D.H., Corbet N.J., Burns B.M., Moore S.S. & Boe-Hansen G.B. (2014) Sperm protamine deficiency correlates with sperm DNA damage in *Bos indicus* bulls. Andrology 2, 370-8.
- Fortes M.R.S., Reverter A., Kelly M., McCulloch R. & Lehnert S.A. (2013) Genome-wide association study for inhibin, luteinizing hormone, insulin-like growth factor 1, testicular size and semen traits in bovine species. Andrology 1, 644-50.
- Fortes M.R.S, Reverter A., Hawken R.J., Bolormaa S. & Lehnert S.A. (2012) Candidate genes associated with testicular development, sperm quality, and hormone levels of inhibin, luteinizing hormone, and insulin-like growth factor 1 in Brahman bulls. Biology of Reproduction 87, 58.
- Fortes M.R.S., Holroyd R.G., Reverter A., Venus B.K., Satake N. & Boe-Hansen G.B. (2012) The integrity of sperm chromatin in young tropical composite bulls. Theriogenology 78, 326-33.
- Lyons R.E., Nguyen To L., Dierens L., Fortes M.R.S., Kelly M., McWilliam S.S., Li Y., Bunch R.J., Harrison B.E., Barendse W., Lehnert S.A. & Moore S.S. (2014) Evidence for positive selection of taurine genes within a QTL region on chromosome X associated with testicular size in Australian Brahman cattle. BMC Genetics 15, 6.

9. Acknowledgements

We acknowledge the efforts of a large number of collaborators that worked together during the CRC for Beef Genetic Technologies and created the vast phenotype resources that made this study possible. Dr Richard Holroyd, Dr Jim Walkley, Dr Brian Burns and Dr David Johnston were instrumental in designing and resourcing the phenotype collections. The Northern Pastoral Group, the Queensland government (DEEDI) and CSIRO collaborated to collect phenotypes. We thank colleagues who contributed as custodians of data and/or samples and performed sperm morphology analyses: Dr Nick Corbet, Ms Debra Corbet and Ms Bronwyn Venus, also Dr Russell Lyons who assured chip genotyping was performed by the Animal Genetics Laboratory (AGL) at the University of Queensland.

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Boe-Hansen G.B., Christensen P., Vibjerg D., Nielsen M.B.F. & Hedeboe A.M. (2008) Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. *Theriogenology* **69**, 728-36.

Browning B.L. & Browning S.R. (2009) A Unified Approach to Genotype Imputation and Haplotype-Phase Inference for Large Data Sets of Trios and Unrelated Individuals. *American Journal of Human Genetics* **84**, 210-23.

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