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Markers and genes influencing puberty

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Abstract

Reproductive traits are moderately heritable traits in beef cattle, which makes genetic selection for components of reproductive rate a promising strategy for beef production in northern Australian herds. The beef industry is currently investing in the development of genomic methods to facilitate more accurate selection decisions. This project examined whether genetic markers developed for the prediction of reproductive traits in beef cows would have predictive value for reproductive traits in bulls, and whether the Sperm Chromatin Structure Assay (SCSA) would be a useful and cost-effective method to monitor onset of puberty in bulls.

Brahman bulls (1,115) with extensive reproductive phenotypes were genotyped using a panel of 50,000 single nucleotide polymorphism (SNP) markers. Associations between male reproductive traits and SNP markers were calculated, and compared with the associations discovered in a related female population. The results indicate that using genetic markers to select for components of reproductive performance in cows is not expected to have a detrimental effect on bull fertility. For example, genetic markers that are associated with age at puberty in heifers show the same association with age at puberty in bulls.

SCSA results were correlated with the percentage of normal sperm, but not with age at puberty. SCSA could be integrated into future breeding soundness evaluation schemes for bulls and has potential to make sperm morphology assessment more cost-effective.

Executive summary

Project Objectives

Brahman cattle predominate in the major beef-producing regions of Australia, as this breed is better adapted to the tropical climate, tropical native pastures and forages and parasite challenges. However, the lower reproductive rate of Brahman cattle is a major contributor to the lower productivity of beef cattle herds in tropical Australia.

The Beef CRC has shown that age at puberty is positively correlated with other fertility traits in the female such as post partum anestrous interval (PPAI) and is highly heritable in Brahman and other *Bos indicus* related breeds of cattle. Selection for animals that are likely to have offspring at an earlier age is therefore a feasible strategy in cattle. However, age at puberty and other reproductive traits are difficult traits to measure. If genetic selection for fertility traits is to have an impact, the industry requires methods that would enable identification of animals with these desirable traits.

The Beef CRC has invested considerable resources into developing genetic markers or genome-based prediction equations for female reproductive performance traits. This project was designed to test whether selection of animals based on genomically-predicted female reproductive performance is going to have a negative effect on male reproductive performance.

Brahman bulls (1,115) with measures of reproductive parameters from the Beef CRC were genotyped with 50,000 single nucleotide polymorphism (SNP) markers (Bovine SNP50 chip). A genome-wide association study was performed and the results were compared with a genome-wide association study previously completed on female Brahman cattle.

An additional aspect of the project was to evaluate the Sperm Chromatin Structure assay (SCSA) as a possible early indicator of bull puberty. SCSA can be performed on frozen semen samples and uses a flow cytometer for its readout. The SCSA was carried out on 3 separate semen samples, from 20 Tropical Composite bulls, to determine the feasibility of using assay results to identify bulls with early puberty or high fertility.

Significant Results and Conclusions

The comparison of gene marker effects in male and female Brahman cattle show that the use of gene markers to select bulls for the predicted reproductive performance of their female offspring will not result in selection decisions that would place herd bulls with poor calf getting ability in breeding herds. On the contrary, components of male and female reproductive performance appear to be tightly linked at the genetic level, so that the marker-assisted selection of animals of either sex for reproductive traits will result in a moderately to strongly correlated response in offspring of either sex.

The genome-wide association study for male reproductive traits has created opportunities for the discovery of causative gene markers for many bull fertility traits, which will contribute to the accuracy of genomic selection for *Bos indicus* breeds. The Beef CRC is about to create further opportunities to interrogate this data set, as much denser genotyping information is currently being imputed for the 1,115 bulls assayed in this project.

When the genetic correlations between male and female reproductive traits were estimated for Brahman bulls, it became apparent that these findings can be used to more effectively select herd bulls in Northern Australia. The most valuable herd bulls for beef production in Northern Australia would lift calf output not only through their own performance as sires but also through producing more fertile daughters in the herd.

The SCSA was found to be 83% correlated ($p<0.01$) with percent normal sperm (PNS, an important predictor of calf output) at 24 months. SCSA was not correlated with age at puberty. Hence, future studies could aim to assess if SCSA could be an aid for predicting calf output under Australian field conditions. As the SCSA relies on flow cytometry, rather than a visual assessment of a microscope image, technology developments could result in higher throughput, improved precision and objectivity compared to sperm morphology assessments.

Recommendations

1. The industry can be confident that the adoption of genomic selection methods to more accurately select bulls for reproductive performance of their female offspring, will not have negative impacts on the reproductive performance of males.
2. Research funding should continue to be directed towards the analysis of the genome-wide association data set, as it is likely to yield information on many important bull fertility genes. One of the goals of future research into this data set will therefore be to identify the gene loci underneath important association peaks herein reported. High density genotyping and genome sequencing data generated during the final year of the Beef CRC will be used towards this end, but work is likely to continue and require funding beyond the conclusion of the Beef CRC.
3. Genetic correlations between male and female reproductive traits enhance selection strategies to more effectively select herd bulls in Northern Australia. The resulting recommendation for the northern industry is to consider a multistage selection protocol that includes established measurements (for example scrotal circumference and sperm morphology assessments) as well as additional assays (serum IGF1 at weaning and genotyping). We can predict that bulls selected in this way will sire daughters that enter puberty earlier and have a shorter PPAI.
4. To further evaluate the SCSA, R&D funding should be invested in measurements from one semen sample at 2 years of age on all the bulls (Brahman and Tropical composite) in the Beef CRC population.

Timeframe and likely beneficiaries from this work

Producers in Northern Australia will be the beneficiaries of the knowledge generated in this project and the wider Beef CRC projects, by being able to lift the calf output from their herds through selecting superior herd bulls.

The impact on herd reproduction rates from the introduction of gene marker information, and other methods of evaluating herd bulls, will not be immediate. Even if gene markers were adopted right now to more accurately select herd bulls for the reproductive performance of their female offspring, their benefit would take a number of years to become apparent as increased calf output from the female offspring of those bulls.

Contents

1 Background

1.1 The relevance of reproductive traits for beef cattle production

Brahman and Tropical Composite breeds predominate the tropical and subtropical regions as they are better adapted and are more resistant to heat stress than *Bos taurus* breeds. Moreover, *Bos indicus* and *Bos indicus* infused cattle are productive on low-quality forage diets and are more resistant to both ecto- and endoparasites. However, cattle productivity in the tropics is generally lower than in temperate regions and delayed age at puberty is a substantial contributor to the low efficiency of beef production in tropical regions.

In contrast to *Bos taurus* bulls that attain puberty around 8–10 months of age and are used for breeding purposes as yearlings, *Bos indicus* bulls generally do not reach puberty until 16–18 months of age and tend to be used as breeding bulls when 2 years old. Selection for earlier age at puberty in *Bos indicus* and crossbred bulls can decrease production costs, reduce generation interval, and increase genetic gains and overall productivity. A similar scenario is observed when comparing *Bos indicus* cows with *Bos taurus* cows.

Puberty is hard to measure and therefore it is difficult to make significant improvements using traditional selection approaches. The identification of genetic markers for the selection of early pubertal animals is an alternative approach for the improvement of this trait in the industry, with the proviso that it does not antagonistically impact on other traits such as survivability of the cow and/or calf and lifetime reproductive performance.

1.2 Genetic markers to predict female reproductive performance

The Beef CRC, with support from MLA, has conducted a genome wide association study using 50K single nucleotide polymorphism (SNP) data on the 'Lifetime Reproductive Performance' cow population in an effort to identify genetic markers for age at puberty and post partum anestrous interval (PPAI). This previous work has identified many chromosomal regions associated with female cattle puberty and length of post partum anestrous interval in both Brahman and Tropical Composite breeds (Hawken, Zhang et al. submitted) .

In this project, we examine the effect of female reproductive gene markers on male reproductive performance. The question posed was whether genetic markers which may be used to predict female reproductive performance would influence phenotypes associated with male reproductive performance. The main objective was therefore to genotype markers discovered for the improvement of female reproductive performance on a population of male cattle that have been extensively phenotyped for male reproductive traits. This population of male cattle was established by Dr. Richard Holroyd (DEEDI), in the Beef CRC Project 4.1.3a 'Male indicator traits of female reproductive performance'. Phenotypic traits measured on this population include sperm motility, morphology, volume and density, scrotal circumference and insulin-like growth factor 1 (IGF1) serum concentrations.

1.3 Reproductive traits measured in young bulls

Fertile young bulls are the main product of stud breeders, but they are equally as important to the commercial producers that buy them. Young bulls are the source of new genetics for the herd and they contribute to the overall reproduction rate. Thus there is value in defining what traits to measure and to incorporate in the selection programs of young bulls.

Ideal traits for selection would be measured early in life (at weaning or yearling) and would be correlated with future mating performance (i.e. calf output). Between weaning and mating bulls

will undergo puberty, a developmental process that leads to the ability to reproduce (Senger 1999). *Bos indicus* bulls, such as Brahman bulls, are late reaching puberty when compared to most other breeds and this genetic trait is associated with reproductive inefficiency in tropical regions (Lunstra, Ford et al. 1978; Lunstra and Cundiff 2003; Abeygunawardena and Dematawewa 2004). Puberty in bulls is currently defined by scrotal circumference thresholds and is also related to sperm concentration, motility and morphology (Table 1) (Lunstra, Ford et al. 1978; Bagu, Madgwicket al. 2004; Siddiqui, Bhattacharjee et al. 2008). Sperm morphology, namely the percentage of normal sperm (PNS), is considered one of the best predictors of calf output for bulls in multiple sire herds (Holroyd, Doogan et al. 2002).

Table 1. Definitions for pubertal phenotype in bovine males found in the literature

Author, Year	Definitions of puberty as a phenotype
Lunstra *et al.* (1978)	50 million sperm cells/ejaculate, minimum 10% motility
Chase *et al.* (1994)	40 million sperm cells/ejaculate, minimum 10% motility
Tatman *et al.* (2004)	50 million sperm cells/ejaculate
Bagu *et al.* (2004)	Scrotal circumference greater than 28cm
Brito *et al.* (2004)	50 million sperm cells/ejaculate, minimum 10% motility

In this study, we aimed to estimate genetic correlations and heritabilities, as well as identify genes and markers associated with desirable traits for the selection of young Brahman bulls. The traits chosen for investigation were weaning weight (WWT), flight time (FT), IGF1 serum levels at weaning (IGF1), yearling weight (WT12), yearling scrotal circumference (SC12), age of achieving a SC of 26 cm (AGE26) and percentage normal sperm measured at 24 month of age (PNS). Traits measured at weaning or yearling would be desirable for being measured early in life, while AGE26 was selected as a threshold SC for puberty and PNS represents a trait associated with calf output.

The Sperm Chromatin Structure Assay (SCSA) was used to measure DNA integrity throughout pubertal development of bulls as we tested this methodology as an additional potential early indicator of male fertility. The SCSA measures DNA integrity in sperm cells. It is known that this measure is highly correlated to fertility in mature male pigs (Boe-Hansen, Christensen et al. 2008). This technology was never before used to evaluate semen from young bulls. Here, a pilot study was performed to examine the utility of the SCSA on further defining puberty and predicting fertility in young bulls.

2 Project objectives

(1) Genotyped 1,000 bulls from the Beef CRC's project 4.1.3a for the top 96 gene markers influencing female reproductive traits, and statistically analysed the association of each gene marker with each male reproduction trait.

(2) To determine and test the utility of the Sperm Chromatin Structure Assay as an early indicator of fertility and pubertal development in bulls on a subset of semen samples collected throughout pubertal development.

3 Methodology

3.1 Animals and traits

Tables summarising the number of animals used and descriptive statistics for all measured traits are provided in the results section.

3.1.1 Animals used for genetic analysis

The animals used in this project belonged to the cattle herd bred by the Beef CRC. This herd was described in detail previously (Barwick, Johnston et al. 2009; Barwick, Wolcott et al. 2009; Corbet, Burns et al. 2009; Johnston, Barwick et al. 2009; Johnston, Barwick et al. 2010). From this herd we selected 843 Brahman cows, 866 Tropical Composite cows and 1,115 Brahman bulls to genotype. Genetic analysis and results reported here used data from genotyped cows and bulls.

Tropical Composite (admixture cattle) and Brahman (*Bos indicus*) are both representative breeds of the Northern Australian beef industry. Tropical Composite cows were part of the "Lifetime reproductive performance" population of the Beef CRC and represent 51 sire families. Tropical Composites are admixes of Belmont Red, Charbray, Santa Gertrudis, Brahman crossbreds and Senepol cattle. The Brahman cows also belong to the "Lifetime reproductive performance" population and they reared the Brahman bulls included in this project. The Brahman pedigree consists of over 50 grandsire families; 54 bulls that sired the cows, which were mated to 55 industry sires to produce the bulls used here.

3.1.2 Animals used for SCSA

The 20 Tropical Composite bulls selected for the SCSA were part of a cohort of 120 bulls born at Brian Pastures Research Station (25ºS, 141ºE) in south-eastern Queensland in September and October 2006. These bulls were weaned in March 2007 and then relocated to Brigalow Research Station (24^{o}S, 149^{o}E) in central Queensland.

The 20 bulls were selected from the available 120 on the following criteria: complete bull breeding soundness evaluation (BBSE) measurements, including an ejaculate containing sufficient sperm cells for morphological estimation at 13, 18 and 24 months of age; birth dates as close as possible; and the bulls represented a number of different sires (maximum three bulls per sire in this sample). The ability to produce an ejaculate with sperm cells has been considered a threshold for puberty in bulls (Unaniam 1997) and the 20 bulls included in this experiment were capable of producing 3 ejaculates (at 13, 18 and 24 month of age). Therefore, this experiment focused on the early post-pubertal development of bulls.

3.1.3 Traits measured: Puberty

The traits that defined age of puberty were: age at which the first *corpus luteum* (CL) was detected in cows (AGECL) and age at a scrotal circumference of 26 cm in bulls (AGE26). The first CL was detected by ovarian ultrasound examination carried out regularly; every 4 to 6 weeks, after the cows had reached an mean liveweight of 200 kg (Johnston, Barwick et al. 2009). Age at the first CL was estimated from annotation of the date when the first CL was observed and the date of birth. Scrotal circumference (SC) was measured with a standard metal tape (Fordyce, Entwistle et al. 2006). Following weaning, eight measurements of SC were taken for each bull, at 3 month intervals. Using these repeated measurements for individual regressions, we interpolated the age of the bull when it achieved 26 cm of SC (AGE26, expressed in days). Achieving SC of 26 cm was considered a threshold for puberty in *Bos indicus* bulls. Observational evidence suggests that a 26 cm SC is associated with the ability to produce an ejaculate in our population of Brahman bulls (Dick Holroyd, personal communication). The threshold was different from the classic 28 cm threshold because *Bos indicus* typically present

more elongated scrota and smaller SC when compared to *Bos taurus* (Lunstra, Ford et al. 1978; McGowan, Bertram et al. 2002; Silva, Pedrosa et al. 2011).

3.1.4 Traits measured: Cows

Assessments were conducted at intervals of between 4–6 weeks. All heifers were weighed, ultrasound-scanned for fat depth at the P8 site and body condition scored (Barwick *et al*. 2009b) at the time of ovarian scanning. The date of the first CL for each heifer was then used to identify other measures recorded on the heifer at this time (or within 7 days) and included heifer liveweight at first CL (WTCL) and ultrasound scan P8 fat depth at first CL (FATCL). An additional trait was defined by first identifying the date of the commencement of the first joining period for each heifer cohort and was defined as the observation of CL on the day of scanning closest to the start of the joining period (CLJOIN).

Heifer growth and body composition traits were measured at two time points T1 (mean age 18 month) and T2 (mean age 24 month); as described by Barwick et al. (2009a). These traits included liveweight (LWT), ultrasound-scanned fat depth at the P8 site (SP8) and over the 12/13th rib (SRIB), scanned area of the *M. longissimus thoracis et lumborum* (eye muscle) at the 12/13th rib (SEMA), body condition score (CS), hip height (HH), concentration of IGF1 in serum (IGF1), and mean daily liveweight gain (ADG). ADG was computed by individual animal regressions of liveweight on days for multiple weights recorded during the 6-month period defined for both T1 and T2 (Table 2).

Cows were mated as two-year olds and post partum anestrous interval (PPAI) was estimated by ovarian scanning following the first calving. The binary trait ability to cycle prior to weaning (CW) was defined by the observation of a CL during lactation.

3.1.5 Traits measured: Bulls

At weaning, bulls were weighed (WWT), and flight time (FT) was electronically recorded on exit from the scales; the time taken, in seconds, for an animal to cover a distance of 1.7 m after exiting a weigh crush (Barwick, Johnston et al. 2009; Barwick, Wolcott et al. 2009). To determine serum IGF1 concentrations at weaning, blood samples were collected via jugular venipuncture onto blood spot cards. The IGF1 analysis was conducted by Primegro Limited (Adelaide, Australia) using a commercially available ELISA assay (Diagnostic Systems Laboratories Inc., Webster, TX).

At the mean ages of 12, 18 and 24 months all bulls were evaluated using standard bull breeding soundness evaluation (BBSE) practices (Fordyce, Entwistle et al. 2006). First, bulls were mustered from the paddock in the morning then weighed over a period of 4 hours and liveweight (WT) was recorded using electronic scales. Body condition score (BCS) was visually measured using a 5-point scale (1 emaciated, 5 over-fat). The scrotal sac was palpated and testicular tone (TT) recorded using a 5-point scale (1 very soft, 5 very hard) with a desirable tone in the 3-4 range. Scrotal circumference (SC) was measured with a standard metal tape in centimetres (Lunstra, Ford et al. 1978). Semen was collected using electroejaculation with the sample being assessed immediately post-collection crush-side for mass activity (MA) and progressively forward motility (Mot) using microscopy. Mass activity was assessed on a 5-point scale (0 no swirl, 5 fast distinct swirl with continuous dark waves). Motility was recorded as a percentage (in increments of 5%).

Two sub-samples of semen were stored for further analysis. One aliquot of 20-120 µL was diluted into a 0.2% glutaraldehyde in phosphate buffered saline solution for preservation for an estimation of sperm morphology and the second into a 500 µL cryovial tube and snap frozen in liquid nitrogen for later analysis of sperm concentration and chromatin integrity.

The morphology of 100 sperm cells was determined by examining a thin cover-slip preparation of semen using phase contrast microscopy with a differential interference contrast objective (magnification at x1000). Sperm were classified individually, then allocated into categories: percent normal sperm (PNS); percent abnormal heads (HA); percent having a cytoplasmic droplet (D) or more specifically having proximal cytoplasmic droplet (PD). All sperm morphology assessments were conducted by the same laboratory technician, a sperm morphologist accredited by the Australian Cattle Veterinarians.

3.1.6 Sperm Chromatin Structure Assay (SCSA)

Samples were thawed at 37ºC for 3 min and sperm concentration of each sample was determined using the Countess™ Automated Cell Counter (Invitrogen™, CA, USA). SCSA was conducted according to the protocol described by Evenson and Jost (Evenson, Jost et al. 1999). The SCSA is based on the metachromatic properties of acridine orange to assess chromatin stability. Acridine orange fluoresces green when combined with double stranded (intact) DNA. However, it fluoresces red when combined with single stranded (damaged) DNA. Semen samples were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 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 µL was transferred to a Beckman Coulter test tube (Beckman Coulter Australia Pty. Ltd., Gladesville, NSW). A volume of 100 µL 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 sec, 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). The samples were analysed using a GalliosTM flow cytometer (Beckman Coulter, Inc., Miami, USA). Green fluorescence (FL1) was collected through a 515–545 nm band pass filter, while orange red fluorescence (FL3) was passed through a 655 dichroic filter and subsequently was collected through a 620 nm band pass filter. In addition, red fluorescence (FL4) was collected using a 730 nm dichroic filter followed by a 695 nm band pass filter. We analysed samples on the low flow rate setting. Acquisition of data began exactly 3 min after the addition of acid detergent solution and 10,000 events were collected per sample using the Gallios software. After every six test samples, a reference sample was thawed and analysed to ensure stability of the instrument. The reference sample was an aliquot from the same ejaculate and was used throughout the experiment. Analyses of list mode data were performed using KaluzaTM (Beckman Coulter, Inc., Miami, USA). The percentage of sperm with abnormally high DNA stainability (HDS), the proportions of sperm with DNA fragmentation, collectively termed the DFI, and the standard deviation of DFI for the total sperm population (SD-DFI) was determined for each sample. The DFI value (DFI1) was determined using the FL3 fluorescence for detecting sperm with DNA damage. Additionally, we calculated a second DFI value (DFI2) based on FL4 fluorescence for detecting sperm with DNA damage. Samples from the different bulls and for each time point (12, 18 and 24 month) were processed in random order.

3.2 Genetic correlations and heritabilities

Using the pedigree relationship, heritabilities and genetic correlations were estimated for 7 traits measured in Brahman bulls. Fixed effects included in the model were contemporary group (i.e. group of cattle born in the same year and raised together), herd of origin, age of the dam and the batch of the IGF1 assay. Age (days) of the animal at the time of measurement was used as a covariate for most traits. Solutions to the effects in the model as well as variance components were estimated using VCE v.6 software (Groeneveld and García-Cortés; http://vce.tzv.fal.de).

Genetic correlations and heritabilities for the female traits were produced and reported previously (Johnston et al 2009, 2010).

Correlations between male and female traits were estimated from the effects calculated for gene markers in two genome wide association studies (see Results for further details).

3.3 Genotypes and gene markers

For genotyping, DNA was extracted from blood samples. The BovineSNP50 Bead Chip version 1 (v1) array (Illumina Inc., San Diego, CA) (Van Tassell, Smith et al. 2008; Matukumalli, Lawley et al. 2009) was used to genotype female cattle samples according to the manufacturer's protocols. Repeat samples were included for quality assurance. The Bead Studio software (Illumina Inc., San Diego, CA) was employed to determine the genotype calls. Animals with call rates inferior to 98% were excluded, resulting in 843 Brahman cows. SNP with auto-calling rates lower than 85% and SNP with a minor allele frequency (MAF) < 0.01 were excluded from further analyses. After these edits, the number of gene markers being retained for the genome wide association study was 44,333 SNP.

Bulls were genotyped with the BovineSNP50 Bead Chip v2 array, which has approximately 600 additional SNP when compared to v1. Before the bull genotype data was analysed, missing genotypes were imputed across v1 and v2 arrays using the BEAGLE 3.2 program (Browning and Browning 2010). Imputation resulted in 50,353 gene markers available. Of these markers 43,821 had a MAF > 0.01 and were retained for association tests in Brahman bulls and a second analysis of the AGECL trait in Brahman cows. The extra genotypes from imputation allowed for better resolution, especially in the X chromosome.

3.4 Genome wide association studies

Genome wide association studies (GWAS) were performed for each trait and breed separately. Genotype calls were coded as 0 for the homozygote of the first alphabetical allele (A), 1 for the heterozygote, and 2 for the homozygote of the second alphabetical allele (B). Alleles A and B were defined according to top/bottom rules from Illumina. The effect of each SNP was estimated in turn using the mixed model in Eq.1.

$$y_{ij} = X\beta + Zu + s_{jk} + e_{ij} \quad [1]$$

Where y_{ij} represents the vector of observations from the i-th cow at the j-th phenotype; X is the incidence matrix relating fixed effects in β with observation in y_{ij}; Z is the incidence matrix relating random additive polygenic effects in u with observation in y_{ij}; s_{jk} represents the additive association of the k-th SNP on the j-th phenotype; and e_{ij} is the vector of random residual effects. Fixed effects included in the model were the same as described for the models used to estimate the genetic correlations and h^2. Solutions to the effects in the model as well as variance components were estimated using Qxpac5 (Perez-Enciso and Misztal 2011). Qxpak5 performs a likelihood ratio test (LRT), testing the model versus the model without the SNP; against a chi-squared distribution with 1 degree of freedom and this was done one SNP at a time.

4 Results and discussion

4.1 Descriptive statistics for all traits measured

Table 2. Brief description, means and standard deviations of all traits measured in Brahman female cattle (n=843).

Time[1]	Trait	Description	Mean ± SD
T1	AGE (days)	Mean age at T1	518.00 ± 54.80
	ADG (kg/day)	Mean daily gain	0.61 ± 0.15
	CS (score 1-10)	Condition score[2]	8.30 ± 1.40
	SEMA (cm2)	Scanned eye muscle area	44.10 ± 6.60
	HH (cm)	Hip height	127.40 ± 4.90
	IGF1 (ng/mL)	Serum IGF1 level	182.60 ± 84.30
	SP8 (mm)	Scanned subcutaneous P8 fat	3.70 ± 1.90
	SRIB (mm)	Scanned rib fat	2.00 ± 1.00
	WT (Kg)	Live weight	287.60 ± 43.80
CL	AGECL (days)	Age at first CL	750.60 ± 142.10
	CLJOIN (0/1)	Presence of CL on joining	0.43 ± 0.50
	FATCL (mm)	Scanned P8 fat at first CL	4.47 ± 2.19
	WTCL (Kg)	Live weight at first CL	334.40 ± 44.80
T2	AGE (days)	Mean Age at T2	713.40 ± 60.40
	ADG (kg/day)	Mean daily gain	0.14 ± 0.23
	CS (score 1-10)	Condition score	7.40 ± 1.40
	SEMA (cm2)	Scanned eye muscle area	44.10 ± 8.80
	HH (cm)	Hip height	132.40 ± 4.90
	IGF1 (ng/mL)	Serum IGF1 level	215.40 ± 92.3
	SP8 (mm)	Scanned subcutaneous P8 fat	3.10 ± 1.80
	SRIB (mm)	Scanned rib fat	1.90 ± 1.00
	WT (Kg)	Live weight	320.00 ± 58.70
PPAI[3]	PPAI (days)	Post partum anoestrus interval	180.53 ± 109.71
	CW (0/1)	Ability to cycle prior to weaning the first calf	0.89 ± 0.84

[1]Time: CL stands for *corpus luteum*; T1 was the observation time when cows mean 18 month of age; T2 was the observation time when cows mean 24 month of age and PPAI stands for *post partum* anoestrus interval observed after first calving.
[2]Condition score of females was assessed on a 1-10 scale.
[3]PPAI and CW were assessed by ultrasound detection of CL after first calving and cows were mated as two year olds. For PPAI and CW there were 629 records, since not all cows conceived and calved.

Table 3. Brief description, means and standard deviations of traits measured in Brahman bulls (n=1,115)

Time[1]	Trait	Description	Mean ± SD
weaning	IGF1 (ng/mL)	Serum IGF1 level	510.40 ± 299.23
	WWT (Kg)	Weaning body weight	201.74 ± 32.47
	FT (sec)	Flight time	116.24 ± 62.56
yearling	WT12 (Kg)	Body weight	244.26 ± 34.12
	SC12 (cm)	Scrotal circumference	20.84 ± 2.83
adulthood	PNS (%)	Percentage of normal sperm	73.38 ± 22.20
	AGE26* (days)	Age at 26 cm of SC	555.06 ± 100.52

[1]Time: weaning was at approximately 6 month of age, yearling means approximately 12 month of age and adulthood represents approximately 24 month of age. *AGE26 was not measured at a specific time; rather it results from consecutive measurements used in a regression (see methods).

Table 4. Means and standard deviations for the 20 Tropical Composite bulls measured at three bull breeding soundness evaluations (BBSE) performed at six month intervals.

Trait (n = 20)	12 month	18 month	24 month	All measurements
Age at BBSE (days)	407.90±10.83	566.85±9.76	742.20±10.45	572.32±138.06
Liveweight (WT; kg)	323.50±36.03	402.50±36.39	401.30±43.82	375.77±53.41
Body condition score[1] (BCS; score 1-5)	2.93±0.13	2.88±0.15	2.60±0.22	2.80±0.22
Flight time (FT; sec)	1.41±0.36	2.20±0.64	2.04±0.47	1.88±60.29
Testicular tone (TT; score 1-5)	3.90±0.22	3.95±0.22	3.80±0.41	3.9±0.30
Scrotal circumference (SC; cm)	29.50±2.27	30.83±2.09	30.95±2.11	30.43±2.22
Sperm traits				
Mass activity (MA; score 1-5)	2.03±0.82	3.05±0.90	3.03±0.72	2.70±0.94
Motility (Mot; %)	61.25±23.22	67.50±15.52	80.50±23.56	69.75±22.25
Concentration (Conc; $x10^6$/ml)	91.97±89.59	383.74±234.50	275.07±192.77	250.26±216.8
Percent normal sperm (PNS; %)	65.75±22.11	74.60±12.01	78.95±14.82	73.10±17.47
Head abnormalities (HA; %)	14.20±14.56	14.00±11.50	10.90±14.03	13.03±13.29
Total droplets (D; %)	8.60±9.93	3.90±3.67	5.80±6.49	6.10±7.31
Proximal droplets (PD; %)	6.80±9.11	2.35±2.25	2.9±3.73	4.02±6.07
Sperm Chromatin Structure Assay (SCSA)				
HDS[2] (%)	4.18±4.96	3.02±1.50	2.39±2.04	3.19±3.25
DFI1[3] (%)	6.78±4.56	5.76±6.20	6.93±12.91	6.49±8.55
DFI2[3] (%)	11.40±6.98	12.07±12.67	11.53±15.97	11.67±12.23

[1]Body condition of males was assessed on a 1-5 scale
[2]HDS: High DNA Stainability.
[3]DFI: DNA fragmentation index, measured by two alternative filters for red fluorescence (see methods).

4.2 Genetic correlations and heritabilities

Heritabilities and genetic correlations were estimated for all traits measured in Brahman bulls, except sperm chromatin integrity, where insufficient measurements were taken to estimate these values. Table 5 shows that serum IGF1 levels and liveweight traits are moderately heritable, while age at puberty (AGE26) is highly heritable. Flight time and percent normal sperm are lowly heritable traits.

The results also show that serum levels of IGF1 are correlated with liveweight and reproductive traits. Residual correlations of this trait with all others are very low, indicating that nutritional and other environmental factors have very little impact on these correlations. Scrotal circumference at 12 months is strongly correlated with age at puberty, although environmental factors can significantly impact that correlation. Weaning weight is very highly correlated with liveweight at 12 months, but environment (as shown by residual correlation values) can strongly affect this correlation. Flight time does not correlate with any of the other traits measured.

These results confirm the findings reported previously, that serum IGF1 levels are an indicator of the reproductive potential of cattle (Velazquez, Spicer et al. 2008). Here we show that the IGF1 levels at weaning, which is usually the first selection opportunity for bulls, are a strong indicator of the reproductive potential of the bull, in particular the age at which it will enter puberty. A measure of scrotal circumference at 12 months may offer another selection opportunity, and will also predict bulls that will enter puberty early.

These results are in agreement with published data on the same population and traits from Beef CRC colleagues (Corbet, Burns et al. 2009; Corbet, Burns et al. 2011).

Table 5. Genetic correlations and heritabilities for seven traits measured on Brahman bulls. Genetic correlations are shown above diagonal, heritabilities in the diagonal (grey cells) and residual correlations below diagonal (associated standard errors in parenthesis).

h^2	IGF1	WWT	FT	WT12	SC12	PNS	AGE26
	0.40	0.37	0.16	0.46	0.57	0.32	
IGF1	**(0.07)**	(0.10)	(0.14)	(0.10)	(0.08)	(0.17)	-0.49 (0.08)
	0.06	**0.45**	0.23	0.95	0.34	-0.05	
WWT	(0.07)	**(0.06)**	(0.15)	(0.02)	(0.07)	(0.18)	-0.23 (0.07)
	-0.05	0.03	**0.19**	0.27	0.20	0.15	
FT	(0.05)	(0.06)	**(0.05)**	(0.16)	(0.14)	(0.21)	-0.17 (0.11)
	0.00	0.86	0.02	**0.40**	0.42	0.10	
WT12	(0.07)	(0.02)	(0.05)	**(0.05)**	(0.07)	(0.16)	-0.32 (0.06)
	0.01	0.55	-0.04	0.60	**0.54**	0.39	
SC12	(0.08)	(0.06)	(0.06)	(0.05)	**(0.06)**	(0.17)	-0.94 (0.03)
	0.04	-0.06	0.00	-0.04	-0.08	**0.18**	
PNS	(0.06)	(0.08)	(0.05)	(0.07)	(0.08)	**(0.07)**	-0.43 (0.15)
	0.06	-0.72	0.01	-0.77	-0.48	-0.06	
AGE26	(0.11)	(0.07)	(0.07)	(0.06)	(0.08)	(0.10)	**0.71 (0.06)**

Table abbreviations: h^2 = heritability, IGF1 = serum IGF1 at weaning, WWT = liveweight at weaning, FT = flight time at weaning, WT12 = liveweight at 12 months, SC12 = scrotal circumference at 12 months, PNS = percent normal sperm at 24 months, AGE26 = age at which a scrotal circumference of 26 cm was reached. Please refer to Methods section and Table 3 for description of measurements and traits.

4.3 Genome wide association study

The results for genome wide association studies are typically presented in Manhattan plots, where chromosomal positions are on the x-axis and the estimated significance (–log(*P*)) for each gene marker are on the y-axis. In these plots, clusters of markers with high –log(*P*) values are visualised as association peaks that indicate chromosomal regions and genes associated with the trait investigated.

4.3.1 Gene markers associated with age at puberty and other phenotypic traits in Brahman bulls

As a first step towards investigating the feasibility of using SNP data to predict male reproductive phenotypes, a genome-wide association study was performed with 50k SNP genotypes of the 1,115 bulls available for this study. This genotype data was acquired prior to imputation of genotypes (see section 3.3). Therefore, resolution for the X chromosome was not sufficient and this chromosome was excluded from Manhattan plots.

In Figure 1 the Manhattan plot for association of gene markers with the puberty trait AGE26 is shown alongside the scrotal circumference at 12 months (SC12) and serum IGF1 levels at weaning (IGF1). An association peak relating to markers on chromosome 14 is evident for all three traits. The most significant associations are observed between serum levels of IGF1 at weaning and markers on chromosome 14.

Figure 2 plots the SNP effect for percentage of normal sperm measured at 24 month of age (PNS); flight time (FT); weaning weight (WWT) and weight measured at 12 month of age (WT12). Significant associations of SNP markers with these traits are observed throughout the genome plot. The live weight traits WWT and WT12 show an association peak at chromosome 14, but this is not apparent for the PNS and FT traits.

The plots further show that many SNP markers across the genome are associated with the traits in question (-log (*P*) > 3), confirming the findings from other genome-wide association studies performed by the Beef CRC and others (refs), that the heritable component of most traits is linked to many markers throughout the genome. This is in contrast to past expectation that few markers of large effect would explain a large component of the genetic variation in a trait.

In practice, this means that SNP data at many different loci need to be used to predict performance or genetic merit of an animal. This observation lends further support to our decision to use 50 k SNP data in this study, rather than a panel of 96 SNP markers, as originally planned.

The observation that variation at a distinct site on chromosome 14 is strongly associated with IGF1 serum levels, as well as live weight and puberty traits, points to a metabolic or physiological phenotype which, at least in part, defines the growth rate and sexual maturation of Brahman bulls. This observation confirms the results of the quantitative genetic analyses, which show significant correlations between serum IGF1 levels and aspects of reproductive fitness in beef bulls (section 4.2).

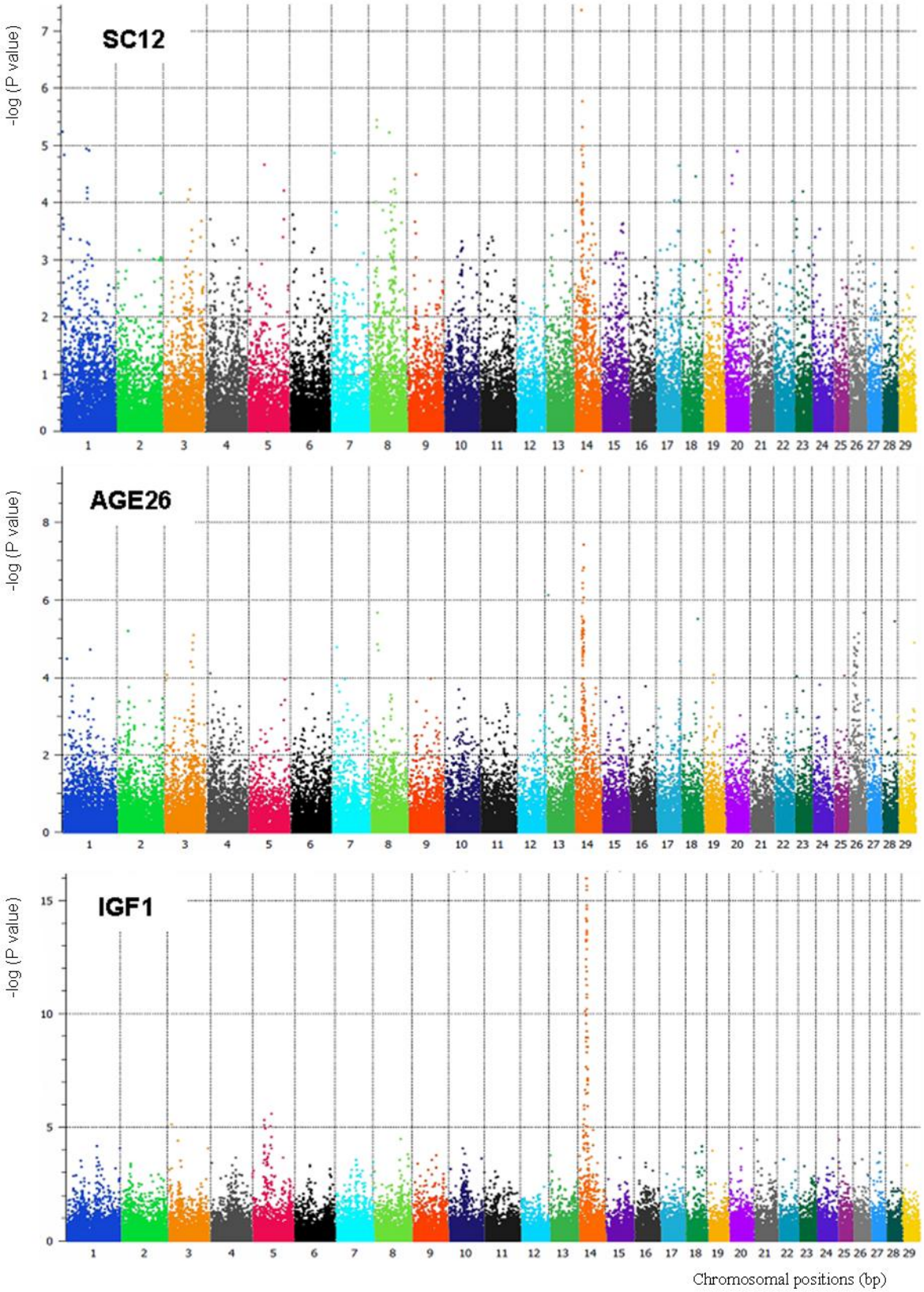

Figure 1: Manhattan plots for scrotal circumference as yearling (SC12), age at puberty (AGE26) and IGF1 (IGF1 serum levels at weaning) measured in Brahman bulls.

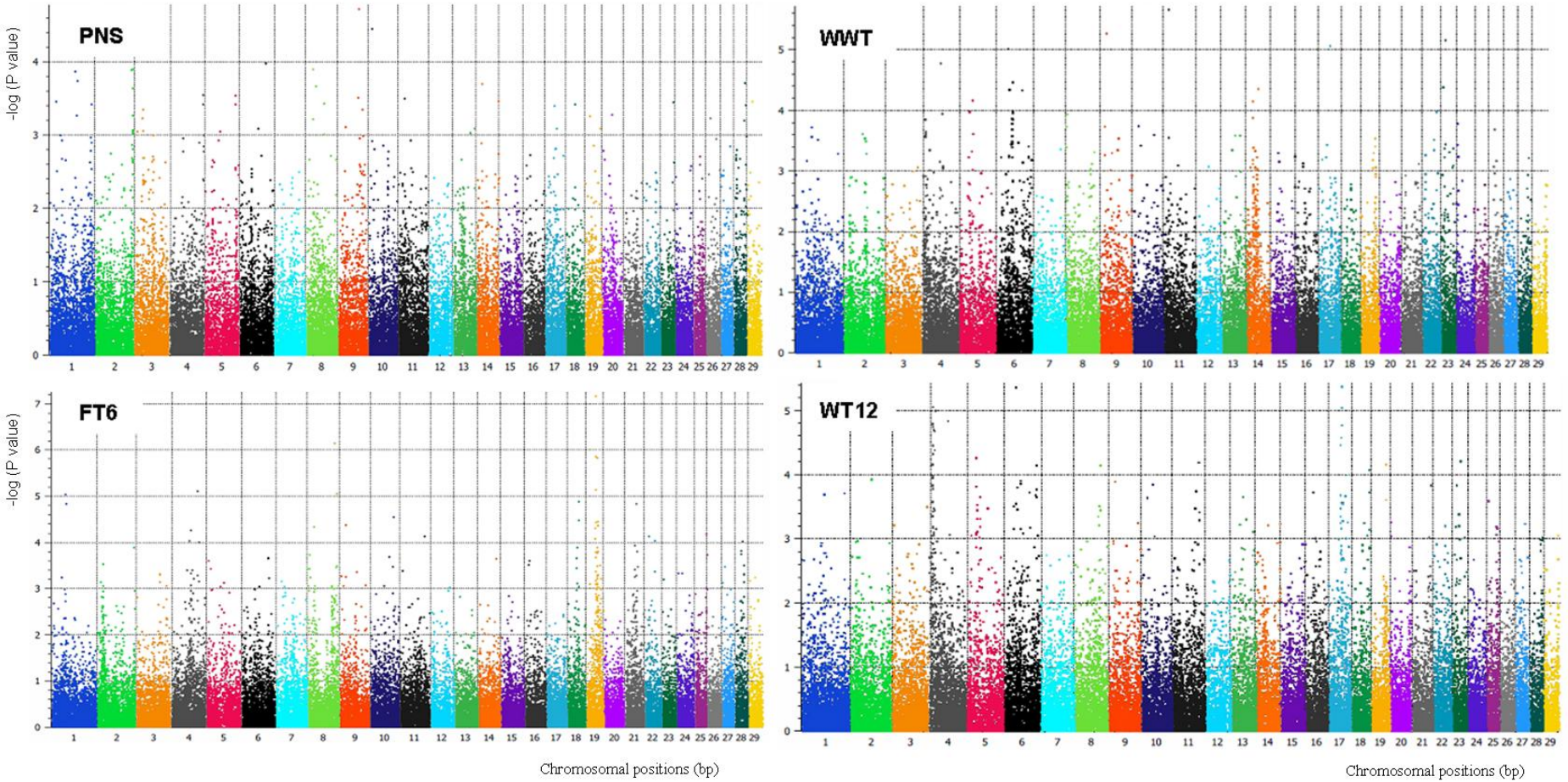

Figure 2: Manhattan plots for percentage of normal sperm measured at 24 month of age (PNS);
flight time (FT); weaning weight (WWT) and weight measured at 12 month of age (WT12) measured in Brahman bulls.

4.3.2 Gene markers associated with puberty in Brahman bulls and heifers

Imputation of missing genotypes across v1 and v2 arrays using the BEAGLE 3.2 program resulted in additional gene markers available for analysis. The extra genotypes from imputation allowed for better resolution, especially in the X chromosome. These genotypes were used to re-analyse the puberty traits AGE26 (Brahman bulls) and AGECL (Brahman heifers). The Manhattan plots resulting from this analysis show that both traits are associated with genetic variation at sites across the entire genome. Of note are the association peaks on chromosome 14 in both sexes. In bulls, genetic markers on the X chromosome also show a highly significant association with puberty.

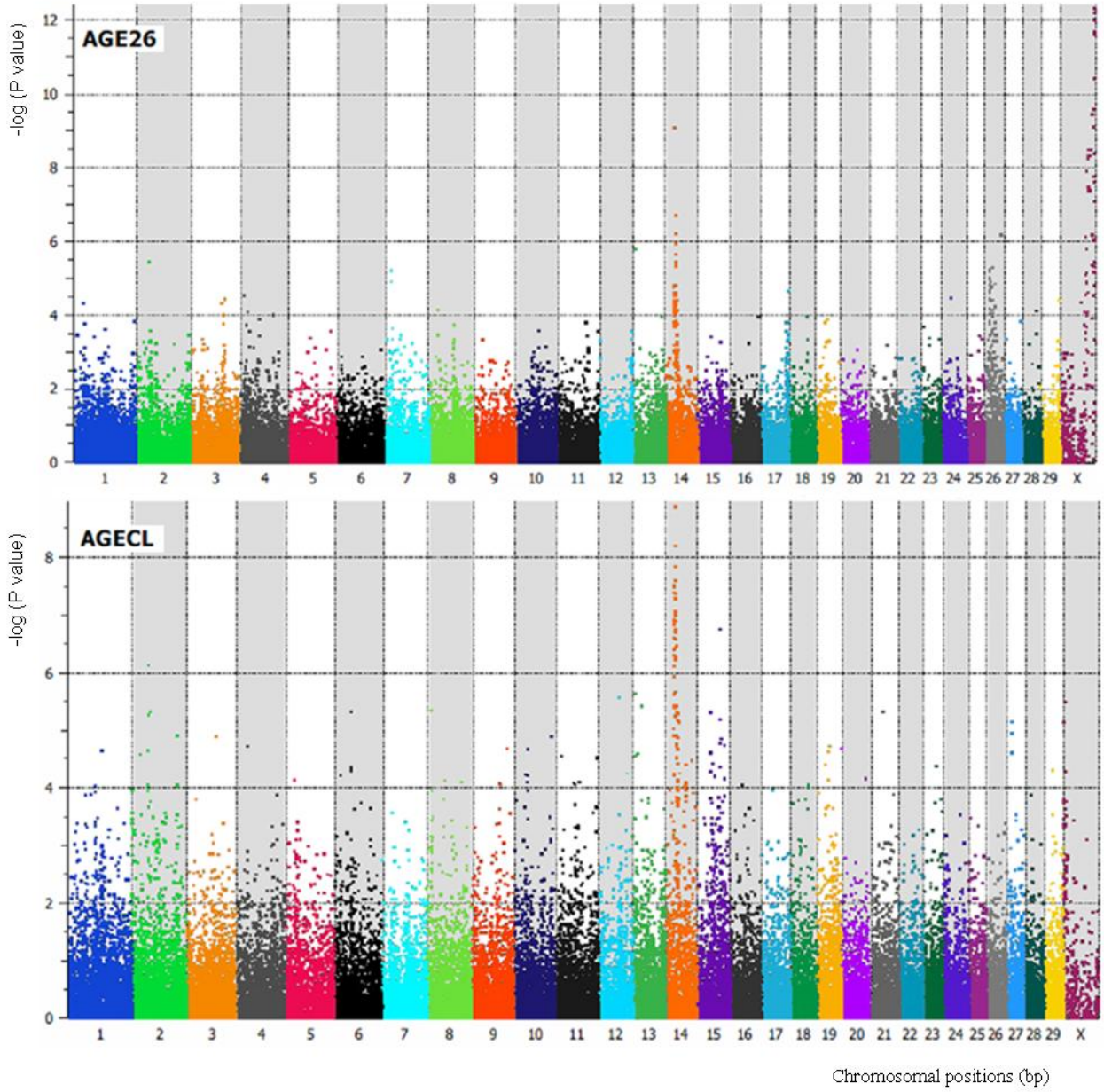

Figure 3: Manhattan plots for puberty in Brahman bulls; age at scrotal circumference 26 cm (AGE26) and heifers; age at which the first *corpus luteum* was detected in cows (AGECL).

4.3.3 Gene markers associated with *post partum* anestrous interval and serum IGF1 levels in Brahman females

Figure 4 illustrates that the association peak on chromosome 14 for Brahman puberty is also observed when SNP markers are plotted against *post partum* anestrous interval (PPAI) and the measurement of IGF1 serum levels at weaning (T1 IGF1) in Brahman females. The association peak on chromosome 14 is not observed in Tropical Composite females (Hawken et al. 2011). This finding lends further support to the notion that IGF1 serum levels at weaning may indicate a metabolic or physiological phenotype that is predictive of reproductive success in Brahman cattle.

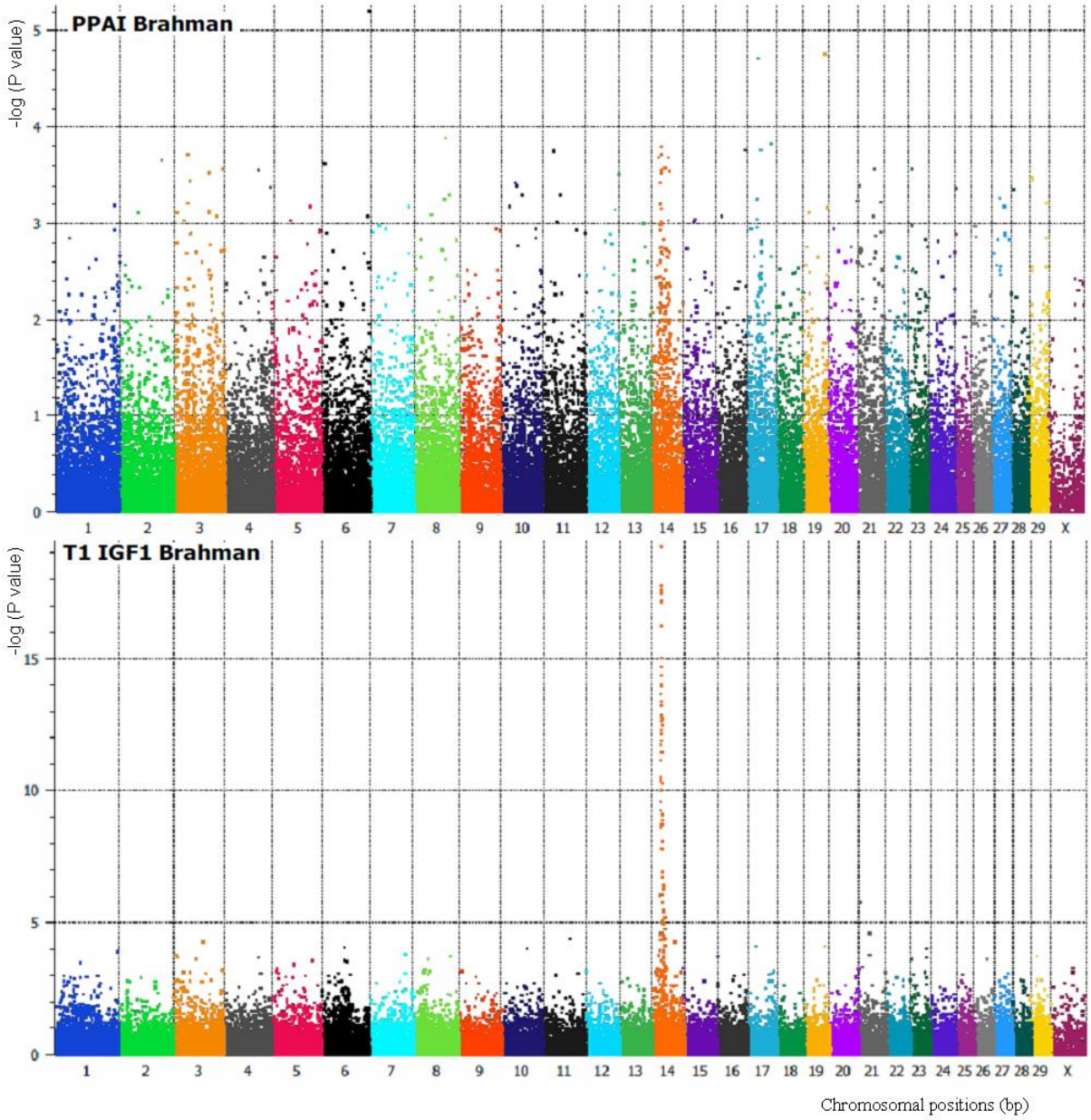

Figure 4: Manhattan plots for post partum anoestrus (PPAI) and serum IGF1 at weaning (T1IGF1) in Brahman heifers and cows.

Knowledge of causative genes underlying a trait can help improve the power of genomic selection, particularly when predictions are applied across different breeds (de Roos, Hayes et al.

2008). One of the goals of future research into this data set will therefore be to identify the gene loci underneath important association peaks.

In order to test whether the association peak observed at chromosome 14 maps to the same genome region for all the traits studied, more detailed plots for chromosome 14 only were examined (data not shown). Our preliminary analysis shows that the chromosome 14 association peak observed for serum IGF1, live weight and age at puberty traits in male and female cattle maps to a gene region that has recently been implicated in cattle reproductive (Pausch, Flisikowski et al. 2011) and live weight (Karim, Takeda et al. 2011) traits in dairy cattle. We are currently investigating the precise location and nature of the significant SNP in our analysis in order to determine whether a gene with a plausible link to these multiple effects can be identified in this region. Progress towards this goal will be documented in the final report for MLA NBP.0364.

The mammalian X chromosome contains many genes of crucial importance for spermatogenesis. Since active spermatogenesis is one of the main factors influencing testis volume, the association peak on the X chromosome linked to a trait based on measurements of scrotal circumference is not completely surprising. Our data now gives us the opportunity to carry out more detailed mapping of the X chromosome with respect to bull reproductive traits and may help identify important causative genes for bull fertility.

4.4 Correlations between SNP effects in bulls and cows

The Beef CRC has invested considerable resources into phenotyping and genotyping beef cows for lifetime reproductive performance. As a consequence, genetic markers or genome-based prediction equations are more likely to be developed for female reproductive performance traits than for males. It is important to test whether selection of animals based on predicted female reproductive performance is going to have a negative effect on male reproductive performance. To test this, statistical analyses were carried out to determine the effects of selecting male cattle based on genetic markers for female reproduction.

To this end, SNP effects from a genome-wide association study (performed by Beef CRC team members at AGBU) on 22 traits measured in Brahman cows (N = 843) were correlated with SNP effects from the GWAS on 7 traits measured in Brahman bulls (N = 1,115). Correlations were calculated using:

- All SNP with SNP effects available in both populations (~ 37K SNP depending on the pair of traits under scrutiny) as well as
- Only those with P < 0.01 in the Brahman cows (~ 600 SNP)
- Only those with P < 0.001 in the Brahman cows (~ 150 SNP)

The above strategy is justified because genomic selection on cows is likely to be driven mostly by significant SNP while the remaining SNP would only contribute “noise”.

Table 6 shows that:

1. Correlations between equivalent traits in cows and bulls were moderate (r ~ 0.5 for body weight) to strong (r ~ 0.8 for serum IGF1).
2. Cow puberty (as measured by Age at 1st CL) showed a 0.6 correlation with bull puberty (as measured by Age at 26 cm of SC).
3. Measures of IGF1 and fat depths in cows (insert traits) were positively correlated with male fertility traits as measured by SC at 12 months (r ~ 0.7) and percent normal sperm at 24 months (r ~ 0.4).
4. PPAI did not show any correlation of relevance with any of the bull fertility traits of interest and only a small negative (yet favourable) with PNS at 24 months (r = –0.14).

In conclusion, marker-assisted selection for fertility traits in cows is not expected to have detrimental effects on bull fertility. In fact, it may actually result in a favourable correlated response. For example, if a SNP-based assay was used to select for females with a shorter PPAI, the correlated response in their male offspring would be a small favourable response in the PNS trait. In another example, selection of breeding cattle with SNP markers for early female puberty will result in a higher likelihood of puberty occurring at an earlier age in both their male and female offspring.

Table 6: Correlations between SNP Additive Effects

		Bull traits						
		AGE26	**FT**	**IGF1**	**PNS**	**SC12**	**WT12**	**WWT**
Cow traits	**T1ADG**	-0.549	-0.380	-0.225	-0.210	0.576	-0.013	-0.138
	T1CSN	-0.291	0.136	0.307	-0.105	0.398	0.037	-0.163
	T1SEMA	-0.200	-0.229	0.296	0.196	0.452	0.450	0.240
	T1HH	0.120	-0.132	-0.543	-0.339	-0.127	0.522	0.610
	T1IGF	-0.761	-0.172	0.745	0.262	0.693	-0.567	-0.630
	T1SP8	-0.696	-0.111	0.761	0.486	0.592	-0.591	-0.659
	T1SRIB	-0.622	0.107	0.497	0.104	0.624	-0.319	-0.397
	T1WT	-0.228	-0.332	-0.293	-0.568	-0.134	0.472	0.558
	AGECL	0.604	0.144	-0.514	-0.195	-0.607	0.195	0.345
	CLJOIN	-0.450	-0.099	0.363	-0.339	0.472	-0.025	-0.136
	FATCL	-0.039	0.024	0.101	0.120	-0.265	0.061	-0.074
	WTCL	0.478	-0.007	-0.561	0.001	-0.425	0.684	0.683
	T2ADG	-0.072	-0.361	0.092	0.048	0.260	0.101	0.230
	T2CS	-0.597	0.013	0.552	-0.097	0.562	-0.185	-0.344
	T2SEMA	-0.312	-0.522	-0.030	0.008	0.250	0.219	0.282
	T2HH	0.378	-0.152	-0.492	-0.429	-0.397	0.591	0.617
	T2IGF	-0.777	-0.111	0.652	0.398	0.722	-0.082	-0.106
	T2SP8	-0.761	-0.168	0.520	0.473	0.710	-0.348	-0.545
	T2SRIB	-0.796	-0.379	0.675	0.476	0.795	-0.380	-0.513
	T2WT	-0.311	-0.139	-0.005	-0.353	0.239	0.509	0.527
	PPAI	-0.050	0.305	0.089	-0.138	0.059	0.124	0.221
	CW	0.087	-0.560	-0.148	-0.009	-0.069	-0.445	-0.434
	T1ADG	-0.549	-0.380	-0.225	-0.210	0.576	-0.013	-0.138
	T1CSN	-0.291	0.136	0.307	-0.105	0.398	0.037	-0.163

For trait definitions, please refer to section 3.1

4.5 SCSA

Table 7 shows the correlations between SCSA measurements at three time points (13, 18 and 24 months) with the measures established at the first bull breeding soundness examination (BBSE) for the 20 bulls (24 months).

The measures of DNA fragmentation index (DFI1 and DFI2) at all time points were significantly correlated with percent normal sperm (PNS) and percentage of head abnormalities (HA) at BBSE. The highest correlation (0.94) was seen between DFI2 and HA at 24 months. DFI1 and DFI2 were very highly correlated at all three time points.

These results show that one DFI measurement carried out at 24 months will predict PNS at this time point. PNS is used in the industry as part of BBSE to predict calf-getting potential of a bull. The estimation of PNS is a labour-intensive process requiring specialist microscopy skills and equipment. Here, we provide an indication that a laboratory assay, using an automated cell counter, could be used as a surrogate for the PNS estimate. Further work is required to provide validation on a larger scale, but this finding raises the future prospect of decreased costs and higher throughput of BBSE.

Table 7: Correlations between sperm chromatin fragmentation and traits measured at bull breeding soundness evaluation (Significance: p value < 0.05 (bold); p value < 0.01 (bold and underlined))

	Trait	Age	WT	BCS	FT	SC	Conc	MA	Mot	PNS	HA	PD	HDS	DFI 1	DFI 2
Across time	HDS	-0.22	-0.07	0.00	**-0.32**	-0.20	-0.17	**-0.30**	**-0.38**	-0.06	0.09	0.17			
	DFI 1	0.02	-0.08	-0.16	-0.08	-0.20	-0.06	-0.08	-0.15	**-0.53**	**0.68**	0.16	0.18		
	DFI 2	0.01	-0.08	-0.14	-0.07	-0.23	0.05	-0.06	-0.15	**-0.53**	**0.68**	0.13	0.08	**0.95**	
	Int	0.00	-0.09	-0.19	0.12	0.12	-0.25	-0.21	0.05	0.20	**-0.29**	0.00	-0.14	**-0.26**	**-0.28**
	DC	-0.01	-0.01	0.05	-0.05	-0.11	-0.11	-0.07	-0.19	**-0.27**	**0.34**	0.20	**0.39**	0.22	0.20
	Frag	0.00	0.11	0.19	-0.12	-0.09	**0.35**	**0.28**	0.04	-0.10	0.17	-0.10	-0.03	0.19	0.23
T1 (13 months)	HDS	0.04	0.24	-0.14	-0.41	-0.25	-0.32	**-0.52**	**-0.61**	0.04	0.06	0.08			
	DFI 1	0.22	-0.01	**-0.60**	-0.41	-0.12	-0.17	-0.31	**-0.51**	**-0.55**	**0.57**	**0.56**	0.42		
	DFI 2	0.18	-0.07	**-0.60**	-0.40	-0.10	-0.03	-0.27	**-0.45**	**-0.62**	**0.62**	**0.56**	0.24	**0.95**	
	Int	-0.18	-0.09	-0.04	0.33	0.09	0.05	-0.12	0.10	0.37	**-0.63**	-0.19	-0.44	**-0.49**	-0.40
	DC	-0.05	-0.01	0.21	-0.26	-0.17	-0.06	-0.04	-0.21	-0.37	**0.47**	0.39	**0.55**	0.38	0.30
	Frag	0.31	0.14	-0.16	-0.21	0.04	-0.01	0.22	0.07	-0.16	0.43	-0.11	0.08	0.32	0.27
months	HDS	-0.27	0.11	-0.11	-0.06	0.34	-0.07	0.18	0.12	-0.16	0.17	0.07			

	DFI 1	0.23	-0.14	-0.32	-0.03	-0.26	0.07	0.14	0.12	-0.42	**0.50**	-0.12	-0.23		
	DFI 2	0.28	-0.15	-0.28	-0.07	-0.35	0.14	0.11	0.08	-0.40	**0.49**	-0.12	-0.28	**0.97**	
	Int	0.18	0.41	-0.17	0.35	0.15	-0.39	-0.22	0.26	0.25	-0.11	-0.05	-0.04	-0.15	-0.16
	DC	-0.05	**-0.50**	0.01	-0.01	-0.06	-0.30	-0.17	-0.38	**-0.48**	0.42	0.15	0.45	0.09	0.10
	Frag	-0.18	-0.24	0.18	-0.37	-0.14	**0.53**	0.30	-0.13	-0.08	-0.05	0.00	-0.13	0.12	0.13
T3 (24 months)	HDS	0.32	-0.08	-0.40	**-0.49**	-0.32	0.00	0.06	-0.01	0.01	0.08	0.34			
	DFI 1	0.03	-0.11	-0.07	-0.02	-0.25	-0.09	-0.12	-0.17	**-0.82**	**0.92**	0.05	0.30		
	DFI 2	0.05	-0.13	-0.05	-0.04	-0.27	-0.01	-0.18	-0.18	**-0.83**	**0.94**	-0.02	0.21	**0.98**	
	Int	-0.02	-0.37	-0.26	0.09	0.25	0.04	-0.17	-0.16	0.20	-0.29	0.14	-0.01	-0.35	-0.38
	DC	-0.22	0.41	0.02	0.02	-0.12	0.01	-0.02	-0.08	0.00	0.14	-0.13	0.16	0.28	0.27
	Frag	0.13	0.27	0.32	-0.12	-0.26	-0.05	0.23	0.25	-0.25	0.29	-0.12	-0.07	0.31	0.35

Abbreviations: Age (age at bull breeding soundness evaluation), WT (liveweight), BCS (body condition score), FT (flight time), SC (scrotal circumference), MA (mass activity), Mot (percentage of cells with motility), PNS (percentage of normal sperm cells), HA (percentage of sperm cells presenting head abnormalities), PD (percentage of sperm cells presenting proximal droplets), HDS (high DNA stainability in SCSA), and DFI (DNA fragmentation index, measured by two alternative filters, see methods in SCSA).

5 Success in achieving objectives

5.1 Genotyping of Beef CRC bulls

This objective was achieved to a higher standard than anticipated, due to the opportunity to genotype bulls with the 50K SNP panel, rather than a panel of 96 markers, as documented in the research agreement. The opportunity presented itself as a result of cost decreases in genotyping and the awarding of additional project funding from the Beef CRC, in part due to the quality and depth of male phenotyping.

As a result of the increase in the quality and quantity of the information, we were able to carry out a comprehensive analysis of correlations between all estimated SNP effects in male and female populations.

The acquisition of genome-wide marker information for the reproductive traits measured in Brahman bulls makes sense in the context of the current thinking on the commercialisation of gene markers by the Beef CRC. Instead of a handful of markers predicting a large proportion of the genetic variation in a trait, it has been shown that many markers with small effects are involved. It is therefore anticipated that genomic selection, using information from thousands of SNP markers, will be the way the technology is adopted by industry.

The genome-wide analysis we were able to carry out on the bull population therefore provides valuable information for the future commercial roll-out of the genomic prediction technology to the industry. Two publications describing this work are currently in preparation (Fortes, Lehnert, et al., submitted; and Fortes, Corbet, et al, in preparation).

5.2 SCSA

This objective was achieved, with a subset of semen samples assayed and evaluated as planned. The evaluation has shown the way for future validation of the technique. Since repeated SCSA measurements throughout puberty do not contribute additional information, validation studies can use just one semen sample per bull. This study has highlighted critical parameters measured by the assay that may be linked to bull fertility. A publication has been submitted based on this work (Fortes, Holroyd, et al., submitted).

6 Impact on meat and livestock industry – Now and in five years time

6.1 Testing of SNP markers in bulls

The main benefit from this project will be improvements in the ability to select young bulls for the predicted reproductive performance of their female offspring. The outcomes of this activity will benefit beef producers by increasing the reliability of gene markers identified from female populations for early puberty. By testing the gene markers in males, this project has provided added confidence to this approach. It is now clear that markers for female reproductive performance will not have a negative effect on aspects of male reproductive capacity.

The impact on herd reproduction rates from the introduction of gene marker information to the industry will not be immediate. Even if gene markers are adopted right now to more accurately select herd bulls for the reproductive performance of their female offspring, it will take 5 years to become apparent as increased calf output from the female offspring of those bulls.

6.2 SCSA evaluation

Further benefit may come from the results of the SCSA evaluation. These trials have shown that this assay is a potentially useful indicator of fertility. Additional work is required to validate this finding on a larger scale, and to determine the heritability of the sperm chromatin fragmentation traits measured by SCSA. If large scale validation confirms this finding, SCSA could be applied to directly select young bulls (24 month of age). Again this would have an economic impact by improving reproduction rates.

To realise the impact from this new knowledge further work is required. The evaluation was carried out on 20 Tropical Composite bulls and will need to be repeated on larger numbers, and on additional cattle breeds. Importantly, we have already determined that future studies on SCSA of young bulls have no need for repeated measurements, which would allow the cost-effective analysis of samples collected from thousands of Beef CRC bulls. Studies on the heritabilities of sperm chromatin traits and genetic correlations with other traits would represent highly novel research and will have to be carried out before adoption by industry can be recommended.

7 Conclusions and recommendations

7.1 Adoption of SNP markers in bull breeding

Our data shows that the use of SNP markers to select bulls for the predicted reproductive performance of their female offspring will not result in selection decisions that would place herd bulls with poor calf getting ability in breeding herds. On the contrary, components of male and female reproductive performance appear to be tightly linked at the genetic level, so that the marker-assisted selection of animals of either sex for reproductive traits will result in a moderately to strongly correlated response in offspring of either sex.

Existing knowledge of the genetic link between reproductive traits in males and females is already used by the industry in the recording of scrotal circumference at 12 months of age as a predictor of the fertility of a bull's daughters. Our results have shown that the addition of gene markers into current systems of selecting for reproductive performance will improve the reliability and power of this proven approach.

The data that has been generated by this project offers the opportunity to identify causal genes for components of reproductive efficiency in beef herds. Knowledge of causative genes and mutations can greatly improve the accuracy of genomic prediction, particularly across different breeds. This fine mapping will be underpinned by valuable datasets of high density genotyping and full genome sequences which the Beef CRC will generate in its final year. In order to fully exploit the opportunity, investment in this research has to continue beyond the life of the Beef CRC.

7.2 Exploiting knowledge of genetic correlations in herd bull selection

Our findings and those of other researchers in the Beef CRC (Corbet et al 2009 and 2011) on the genetic correlations and heritabilities of components of reproductive performance in male and female cattle can be used to more effectively select herd bulls in Northern Australia. The most valuable herd bulls for beef production in Northern Australia would lift calf output not only through their own performance but also through siring more fertile daughters. A process of multistage selection of prospective herd or stud bulls could be envisaged:

1. *from birth* - ***genetic markers***: this assumes that the industry has validated marker panels for female reproductive traits available. Marker tests will give the same result no matter at which stage of an animal's life they are applied.
2. *at weaning* - ***IGF1*** **and** ***FT***: traits assessed at the earliest selection opportunity, this will select the animals with the highest likelihood of reaching puberty early, as well as siring fertile offspring, and having the right temperament
3. *at 12 months of age* – ***SC:*** a predictor of the bull's own age at puberty and of the reproductive performance of the bull's female offspring (already in BREEDPLAN).
4. *at 2 years of age* – ***PNS:*** this measure is predictive of the calf getting ability of the bull, already part of the breeding soundness examination prior to first mating (may be assisted by a more cost-effective measure in future, see section 7.3)

The industry already uses components of this process. Both serum IGF1 assays as well as genotyping are currently expensive procedures and therefore not likely to be widely adopted. However, alternative assay technologies for serum IGF1 could be developed, that would bring the cost of the procedure down and improve turnaround time. This development work would require further R&D investment, for example in mass spectrometry-based assay validation.

The development of improved and cost-effective SNP arrays may require further R&D investment. There is already a clear trend of genotyping prices decreasing rapidly, and costs may soon reach a level where the testing of valuable animals such as herd and stud bulls may become feasible. If the cost of genotyping and bioinformatics analysis does not continue to drop sufficiently to make genetic screening of large numbers of calves feasible, cost savings could be achieved by applying genotyping to rank an already select group of bulls at 2 years of age.

7.3 Utility of SCSA in Bull Breeding Soundness Examination

The SCSA measure of DFI is a good predictor of male fertility in mammals (Evenson and Jost 2000; Boe-Hansen, Christensen et al. 2008). Our results show that DFI is correlated with percent normal sperm at 24 months (PNS). PNS is a trait that is significantly correlated with calf output (Holroyd, Doogan et al. 2002). Hence, future studies could aim to assess if SCSA could be an aid for predicting calf output under Australian field conditions. As the SCSA relies on flow cytometry, rather than a visual assessment of a microscope image, technology developments could result in higher throughput, improved precision and objectivity compared to current sperm morphology assessments.

Our recommendation would be to carry out SCSA measurements from one semen sample at 2 years of age on all the bulls (Brahman and Tropical composite) in the Beef CRC population, to further test the strength of the correlation between DFI and PNS, and to be able to calculate heritabilities and genetic correlations for the DFI measurement. Research in this area would guide the industry on the relevance of incorporating the SCSA in the bull breeding soundness evaluation (BBSE).

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Appendices

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Abstract: Sperm chromatin fragmentation is associated with sub-fertility, but its relationship with age progression in young bulls is poorly understood. This study aimed at assessing sperm chromatin fragmentation during early post-pubertal development of 20 tropical composite bulls, using sperm chromatin structure assay (SCSA) and sperm-bos-halomax (SBH). Bulls were subjected to bull breeding soundness evaluation (BBSE) at mean ages of 13, 18 and 24 months. Traits measured included liveweight (WT), body condition score (BCS) and scrotal circumference (SC). Semen samples were collected by electroejaculation and assessed for mass activity (MA), motility (Mot), concentration (Conc), sperm morphology and chromatin fragmentation. Conc ($r = 0.34$, $P = 0.0076$), Mot ($r = 0.36$, $P = 0.0041$) and the percentage of morphological normal sperm (PNS; $r = 0.31$, $P = 0.0132$) were positively correlated with age. The percentage of sperm with proximal droplets (PD) was negatively correlated with age ($r = -0.28$, $P = 0.0348$). SCSA and SBH results showed no correlation with age. The percentage of sperm with chromatin fragmentation using SCSA was correlated with PNS ($r = -0.53$, $P < 0.0001$), the percentage of sperm with head abnormalities ($r = 0.68$, $P < 0.0001$) and the percentage of intact sperm (Int) in SBH ($r = -0.26$, $P = 0.0456$). In summary, sperm chromatin fragmentation was correlated with other reproductive traits and it could be measured in young bulls. For assessment of sperm chromatin fragmentation samples could be equally collected at 13, 18 or 24 month of age as results did not vary with age.

The integrity of sperm chromatin in young tropical composite bulls

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Abstract

Sperm chromatin fragmentation is associated with sub-fertility, but its relationship with age progression in young bulls is poorly understood. This study aimed at assessing sperm chromatin fragmentation during early post-pubertal development of 20 tropical composite bulls, using sperm chromatin structure assay (SCSA) and sperm-bos-halomax (SBH). Bulls were subjected to bull breeding soundness evaluation (BBSE) at mean ages of 13, 18 and 24 months. Traits measured included liveweight (WT), body condition score (BCS) and scrotal circumference (SC). Semen samples were collected by electroejaculation and assessed for mass activity (MA), motility (Mot), concentration (Conc), sperm morphology and chromatin fragmentation. Conc (r = 0.34, *P* = 0.0076), Mot (r = 0.36, *P* = 0.0041) and the percentage of morphological normal sperm (PNS; r = 0.31, *P* = 0.0132) were positively correlated with age. The percentage of sperm with proximal droplets (PD) was negatively correlated with age (r = -0.28, *P* = 0.0348). SCSA and SBH results showed no correlation with age. The percentage of sperm with chromatin fragmentation using SCSA was correlated with PNS (r = -0.53, *P* < 0.0001), the percentage of sperm with head abnormalities (r = 0.68, *P* < 0.0001) and the percentage of intact sperm (Int) in SBH (r = -0.26, *P* = 0.0456). In summary, sperm chromatin fragmentation was correlated with other reproductive traits and it could be measured in young bulls. For assessment of sperm chromatin fragmentation samples could be equally collected at 13, 18 or 24 month of age as results did not vary with age.

Keywords: semen analysis; sperm morphology; sperm chromatin fragmentation; bovine puberty

1. Introduction

The beef cattle industry would benefit from early detection of bulls with reproductive merits or issues. Early-life assessment of reproductive traits has the potential to hasten animal selection decisions and improve genetic gain [5,24]. Therefore, it is relevant to understand the pubertal process and evaluate which reproductive traits can be assessed at a young age.

Puberty is a developmental process in which endocrine and morphologic changes transform the animal into an individual capable of reproducing [26]. The definition of puberty in bulls has been associated with the ability of producing an ejaculate and sexual maturity has been associated with the ability of producing an ejaculate with adequate sperm concentration and motility [2,5,21]. For adequate sperm production, adequate spermatogenesis is necessary. During spermatogenesis chromatin is re-organized into a compact form where sperm DNA is associated with protamins that replace histones. As sperm passes through the epididymis, DNA is further stabilized by disulphate bindings. This complex sperm chromatin packaging is fundamental for its DNA integrity [6]. However, packaging errors and changes in the microenvironment of the epididymis may lead to sperm chromatin fragmentation, which can be measured using the sperm chromatin structure assay (SCSA) and the sperm-bos-halomax (SBH) assay [10,16,23]. The SBH has the advantage of not requiring flow cytometry, but it lacks in robustness because far fewer sperm cells are assessed and with less precision. Sperm chromatin fragmentation has been associated with male sub-fertility and early embryonic death in humans [8,10,20]. In boars, sperm with chromatin fragmentation higher than 2.1% leads to smaller litter sizes [4]. A relationship between sperm chromatin fragmentation and bull fertility, measured by conception after artificial insemination (AI) has also been reported [8,22,28].

Nonetheless, the association between chromatin fragmentation and early post-pubertal development is poorly understood in most species. One study in hamsters showed that sperm DNA from younger animals was less tightly bound to protamine and less fertile [29]. It is unknown if similar results could be expected for bulls or if sperm chromatin fragmentation at a young age correlates to sperm morphology. Sperm morphology was considered one of the best predictors of calf output for bulls in multiple sire herds [16] and so it is of practical relevance to assess correlated traits.

Young bulls starting to produce sperm were reported to have higher percentages of sperm with head abnormalities (HA) and proximal droplets (PD) [5,19,24,30]. In bulls as well as in men, both HA and PD were related to sperm chromatin packaging and fragmentation [9,12,13]. Therefore, we hypothesized that the young post-pubertal bulls, would have higher levels of chromatin fragmentation compared to the mature bulls and that the level of chromatin fragmentation in young post-pubertal bulls may be correlated to sperm morphology. To test this hypothesis we measured sperm chromatin integrity in 20 tropical composite bulls using two different techniques, sperm chromatin structure assay (SCSA) and sperm-bos-halomax (SBH) assay, at 13, 18 and 24 month of age. Sperm chromatin fragmentation values were then correlated with physical, reproductive and seminal traits collected at bull breeding soundness evaluation (BBSE).

2. Materials and Methods

2.1 Animals and Environment

The tropical composite bulls, admixes of Belmont Red, Charbray, Santa Gertrudis and Senepol, were part of a cohort of 120 bulls born at Brian Pastures

Research Station (25ºS, 141ºE) in south-eastern Queensland in September and October 2006. The bulls were weaned in March 2007 and then relocated to Brigalow Research Station (24^{o}S, 149^{o}E) in central Queensland.

The environment at Brigalow is sub-tropical with average annual rainfall of 730 mm with about 50% occurring between November and February. Mean minimum and maximum temperatures range from between 21° and 33°C for January to between 4° and 22°C for July. The paddocks were undulating, with shade from native trees. The pastures were a mixture of Buffel grass (*Cenchrus ciliaris*), Bluegrass (*Dicanthium sericeum*), Rhodes grass (*Chloris gayana*), Green panic grass (*Panicum maximum*) and Seca stylo (*Stylosanthes scabra*). Stocking rate varied but overall averaged 1 adult equivalent (AE) to 3 ha.

2.2 Physical and reproductive examination of bulls

At a mean age of 13 months (T1, November 2007), 18 months (T2, April 2008) and 24 months (T3, October 2008), bulls were mustered from the paddock that morning then weighed over the following 4 hours and liveweight (WT) was recorded using electronic scales. On exit from the scales, flight time (FT) was electronically recorded and defined as the time taken, in seconds, for an animal to cover a distance of 1.7 m after exiting a weigh crush [3].

Over the next 8 days each bull underwent a BBSE based on that of Entwistle and Fordyce [15]. Body condition score (BCS) was visually measured using a 5-point scale (1 emaciated, 5 over-fat). The scrotal sac was palpated and testicular tone (TT) recorded using a 5-point scale (1 very soft, 5 very hard) with a desirable tone in the 3-4 range. Scrotal circumference (SC) was measured with a standard metal tape in centimeters [15].

Semen was collected using electroejaculation with the sample being assessed immediately post-collection crush-side for mass activity (MA) and progressively forward motility (Mot) using microscopy. Mass activity was assessed on a 5-point scale (0 no swirl, 5 fast distinct swirl with continuous dark waves). Motility was recorded as a percentage (in increments of 5%). All of the above described semen evaluation characteristics were based on standard protocols [14,15].

Two sub-samples of semen were stored for further analysis. One aliquot of 20-120 μL was diluted into a 0.2% gluteraldehyde in phosphate buffered saline solution for preservation for an estimation of sperm morphology and the second into a 500 μL cryovial tube and snap frozen in liquid nitrogen for later analysis of sperm concentration and chromatin integrity.

The morphology of 100 sperm cells was determined by examining a thin cover-slip preparation of semen using phase contrast microscopy with a differential interference contrast objective (magnification at ×1000). Sperm were classified individually, then allocated into categories: percent normal sperm (PNS); percent abnormal heads (HA); percent having a cytoplasmic droplet (D) or more specifically having proximal cytoplasmic droplet (PD) [14]. All sperm morphology assessments were conducted by the same laboratory technician, a sperm morphologist accredited by the Australian Cattle Veterinarians [15].

2.3 Selection of bulls for the study

The semen samples of 20 bulls were selected based on the following criteria: complete BBSE measurements including an ejaculate containing sufficient sperm cells for morphological estimation at the three time points; birth dates as close as possible and the bulls represented a number of different sires (maximum three bulls per sire in this sample). The ability to produce an ejaculate with sperm cells has been

considered a threshold for puberty in bulls [27] and the 20 bulls included in this experiment were capable of producing 3 ejaculates (at 13, 18 and 24 month of age). Therefore, this experiment focused on the early post-pubertal development of bulls.

2.4 Sperm chromatin structure assay

Samples were thawed at 37ºC for 3 min and sperm concentration of each sample was determined using the Countess™ Automated Cell Counter (Invitrogen™, CA, USA). SCSA was conducted according to the protocol described by Evenson and Jost [7]. The SCSA is based on the metachromatic properties of acridine orange to assess chromatin stability. Acridine orange fluoresces green when combined with double stranded (intact) DNA. However, it fluoresces red when combined with single stranded (damaged) DNA. Semen samples were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 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 µL was transferred to a Beckman Coulter test tube (Beckman Coulter Australia Pty. Ltd., Gladesville, NSW). A volume of 100 uL 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 sec, 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). The samples were analyzed using a Gallios™ flow cytometer (Beckman Coulter, Inc., Miami, USA). Green fluorescence (FL1) was collected through a 515–545 nm band pass filter, while orange red fluorescence (FL3) was passed through a 655 dichroic filter and subsequently was collected through a 620 nm band pass filter. In addition, red fluorescence (FL4) was collected using a 730 nm dichroic filter followed by a 695 nm band pass filter. We analyzed samples on the low

flow rate setting. Acquisition of data began exactly 3 min after the addition of acid detergent solution and 10,000 events were collected per sample using the Gallios software. After every six test samples, a reference sample was thawed and analyzed to ensure stability of the instrument. The reference sample was an aliquot from the same ejaculate and was used throughout the experiment. Analyses of list mode data were performed using Kaluza^{TM} (Beckman Coulter, Inc., Miami, USA). The percentage of sperm with abnormally high DNA stainability (HDS), the proportions of sperm with DNA fragmentation, collectively termed the DFI, and the standard deviation of DFI for the total sperm population (SD-DFI) was determined for each sample. The DFI value (DFI1) was determined using the FL3 fluorescence for detecting sperm with DNA damage. Additionally, we calculated a second DFI value (DFI2) based on FL4 fluorescence for detecting sperm with DNA damage. Samples from the different bulls and for each time point (T1, T2 and T3) were processed in random order.

2.5 Sperm-bos-halomax assay

Sperm chromatin fragmentation was assessed on the same samples with a sperm-bos-halomax (SBH) kit (ChromaCell SL, Madrid, Spain). The assessments were carried out according to the manufacturer's instructions described for fluorescent microscopy [16]. Briefly, aliquots of agarose gels provided in the kit were liquefied at a temperature over 70^{o}C, and kept at 37^{o}C until used for the assay. The same samples used for the SCSA assay, which were thawed and diluted in TNE buffer, were assessed concurrently. An aliquot of 10 µL of each sample was added to a vial of liquid low melting agarose gel at 37ºC. Then, 25 µL of the sperm cell-agarose suspension was placed onto pre-treated slides, flattened by a coverslip and cooled to 4ºC for a minimum of 5 min. After the resultant gel had solidified, the coverslip was carefully removed. The gels on the slides were immediately placed into a bath of

10 mL of the lysing solution at room temperature for 4 min. The slides were then washed for 5 min in distilled water. The gels were finally dehydrated sequentially in ethanol solutions of 70, 90 and 100% for 2 min each. The slides were stained with 2.5 µL of Gel Red (Sigma-Aldrich Co., Australia), before fluorescent microscopy assessment. The sample used as a reference for SCSA was also assessed with SBH.

Each sample was observed using a fluorescent microscope (Nikon Eclipse E800 coupled with a Nikon digital camera DXM1200F, magnification at x40) and 100 sperm were counted and classified using three categories: 1) Intact sperm (Int) which did not presented any sign of chromatin damage; 2) Sperm which showed some degree of chromatin relaxation or decondencing chromatin (DC); and 3) Sperm with fragmented chromatin (Frag) that exhibited an evident halo.

2.6 Statistical analyses

Correlation analyses were performed using the Procedure CORR of SAS^{TM}, version 9.2 (SAS Institute Inc., Cary, NC, USA). Testicular tone scores were excluded from statistical analysis due to low variability between individuals or time points. Pearson correlations were used to calculate the relatedness between all traits measured. To test the relatedness of BBSE traits and age (for monitoring early post-pubertal development) correlations both across T1, T2 and T3 and within each time point were calculated. Correlations were considered significantly different from zero when $P < 0.05$.

Additionally, in order to ascertain the effect of age on chromatin fragmentation (DFI1, DFI2 and Int) we use the Procedure GLM of SAS^{TM}, version 9.2 (SAS Institute Inc., Cary, NC, USA) to fit a 1-way ANOVA model with time point with three discrete levels (T1, T2 and T3) as the sole independent variable.

3. Results

Means and standard deviations of all traits measured at T1 (average age 13 month or 407.90 ± 10.83 days), T2 (average age 18 month or 566.85 ± 9.76 days) and T3 (average age 24 month or 742.20 ± 10.45 days) are presented in Table 1. Correlations across and within time points for SCSA, SBH and its relationship with other reproductive traits are summarized in Table 2. Correlations between all trait-pairs are provided as supplementary material (Tables S1, S2, S3, and S4).

Across time points, liveweight, SC, sperm concentration, motility and percentage of normal sperm were positively correlated ($P < 0.05$) with age. Body condition score and the percentage of sperm cells with proximal droplets were negatively correlated ($P < 0.05$) with age. There were no correlations between age and sperm chromatin integrity, measured using SCSA and SBH. The 1-way ANOVA model for testing the association between time points and chromatin fragmentation resulted not significant for any of the dependent variables: DFI1 ($P = 0.8986$), DFI2 ($P = 0.9838$) and Int ($P = 0.2267$). The percentage of sperm cells with chromatin fragmentation (DFI1, in SCSA) was correlated with the percentage of normal sperm cells (r = -0.53, $P < 0.0001$), the percentage of cells with head abnormalities (r = 0.68, $P < 0.0001$) and the percentage of cells with intact chromatin in SBH (r = -0.26, $P = 0.0456$). Similar results were obtained for DFI2, as DFI1 and DFI2 were highly correlated (r = 0.95, $P < 0.0001$). HDS was positively correlated with percentage of DC cells (r = 0.39, $P = 0.0021$). HDS was negatively correlated with motility (r = -0.38, $P < 0.01$), mass activity (r = -0.30, $P < 0.05$) and flight time (r = -0.32, $P < 0.05$).

Within each time point, mass activity and motility were positively correlated ($P < 0.05$). The two alternative measurements of DFI (DFI1 and DFI2) were also correlated for within each of the three time points ($r \geq 0.95$, $P < 0.01$).

Chromatin fragmentation measured by SCSA (DFI) was negatively correlated ($r = -0.26$, $P < 0.05$) with the percentage of intact sperm cells observed with SBH. However, this correlation was not high or consistently significant across time points.

Discussion

In this study, and for the first time in cattle, we measured sperm chromatin fragmentation during early post-pubertal development (from 13 to 24 month of age). This period is relevant for bulls since it starts with puberty, defined by the presence of sperm cells in the ejaculate [27], and it coincides with the progression to sexual maturity, defined by sperm concentration and motility thresholds considered adequate for normal fertility [5]. For a complete analysis of the early post-pubertal development, we studied the correlations between SCSA, SBH, all traits routinely measured in BBSE and age at BBSE.

Initially, we hypothesized that young bulls would have higher levels of chromatin fragmentation than mature bulls. This hypothesis was not supported by SCSA and SBH results, which showed no correlations between sperm chromatin fragmentation and age. Meanwhile, other traits measured confirm that the studied period coincided with progression to sexual maturity of bulls. For example, sperm concentration, motility and percentage of normal sperm were positively correlated with age, improving during the studied period. This result agrees with the consensus knowledge about bovine progression to sexual maturity [5,19,24,30]. Brito and colleagues [5] evaluated bulls from 16 to 30 month of age and observed increasing

sperm motility and normal morphology. They also used a threshold percentage of normal sperm (70%) to define sexual maturity in bulls. In our results, the percentage of sperm with proximal droplets was negatively correlated with age. This is also expected since younger bulls commonly present more sperm cells with proximal droplets as a result of abnormal spermiogenesis related to immaturity [1]. In humans cytoplasmic droplets have been associated with enhanced susceptibility of sperm chromatin to fragmentation; [13]. Abnormal levels of cytoplasmic retention, in other words droplets, contain enzymes and support the generation of reactive oxygen species that influences chromatin fragmentation, for a review see [25]. In agreement, we found a correlation between chromatin fragmentation and proximal droplets in young bulls (average 13 month of age), but not in latter evaluations. We could speculate that higher percentages of proximal droplets were the basis for chromatin fragmentation in young bulls, but not at a level that caused young bulls to present significantly higher chromatin fragmentation than mature bulls. In summary, we were able to find the expected associations between age progression and a number of sperm quality traits, but fail to demonstrate an association between age progression and sperm chromatin fragmentation. Therefore, we dispute the hypothesis that young bulls would have higher levels of sperm chromatin fragmentation than mature bulls.

Sperm chromatin fragmentation measured by SCSA was negatively correlated to the percentage of normal sperm cells, across and within time points. Abnormal sperm cells, especially cells with droplets and head abnormalities, have been associated with chromatin packaging and fragmentation [9,12,25]. The nuclear protein of abnormal sperm cells showed a 16 fold increase in binding to actinomycin D, which points to the relationship between abnormal sperm and defects in sperm chromatin packaging [17]. Errors in sperm chromatin packaging may cause chromatin

fragmentation [6]. In this sense, a correlation between SCSA results and sperm morphology was expected.

In this study, SBH results showed inconsistent correlations with percentage of normal sperm, potentially because SBH is a more subjective measurement of chromatin fragmentation and a lower number of sperm cells are evaluated when using this method. This result is in contrast with a previous experiment by Garcia-Macias and colleagues that showed a significant correlation ($r \sim 0.50$, $P < 0.001$) between SBH and the percentage of sperm with head abnormality [16]. Garcia-Macias and colleagues also showed that SBH was not correlated to SCSA results and argued that the two assays could measure different aspects of sperm chromatin. Considering the availability of a flow cytometer and the robustness of the test, SCSA would be the assay of choice for measuring sperm chromatin fragmentation in future studies. As for the use of different detectors in SCSA (FL3 versus FL4), DFI2 (or FL4) gave slightly higher measurements than DFI1 and one could speculate about higher sensitivity of FL4 filter for Acridine Orange fluorescence. However, this difference was not significant and the results for DFI1 and DFI2 were highly correlated. Therefore, either measurement could be used in future studies.

Both SCSA and SBH have been shown to be useful predictors in fertility trials [8,10,11,16,20,28]. For a cattle example, chromatin fragmentation measured by SCSA was significantly associated to AI success [28]. Also, chromatin fragmentation measured by SBH was correlated with non-return rates observed in cows [16]. A different methodology, by the Fuelgen-stain, showed that abnormal chromatin in Nelore bulls over 30 month of age affects *in vitro* embryo production [11]. In the present study, chromatin fragmentation measured using SCSA correlated with sperm morphology, a reported predictor of calf output [18].

In summary, sperm chromatin fragmentation was correlated with important reproductive traits, namely percentage of normal sperm, and it could be measured in young bulls. For assessment of sperm chromatin fragmentation, samples could be equally collected at 13, 18 or 24 month of age for its results did not vary with age.

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Table 1. Means and standard deviations for traits measured at three bull breeding soundness evaluations (BBSE) performed with six month intervals for the same 20 Tropical Composite bulls.

Trait (n = 20)	T1 (Nov-07)	T2 (Apr-08)	T3 (Oct-08)	Across time
Age at BBSE (days)	407.90±10.83	566.85±9.76	742.20±10.45	572.32±138.06
Liveweight (WT; kg)	323.50±36.03	402.50±36.39	401.30±43.82	375.77±53.41
Body condition score (BCS; score 1-5)	2.93±0.13	2.88±0.15	2.60±0.22	2.80±0.22
Flight time (FT; sec)	1.41±0.36	2.20±0.64	2.04±0.47	1.88±60.29
Testicular tone (TT; score 1-5)	3.90±0.22	3.95±0.22	3.80±0.41	3.9±0.30
Scrotal circumference (SC; cm)	29.50±2.27	30.83±2.09	30.95±2.11	30.43±2.22
Sperm traits				
Mass activity (MA; score 1-5)	2.03±0.82	3.05±0.90	3.03±0.72	2.70±0.94
Motility (Mot; score 1-5)	61.25±23.22	67.50±15.52	80.50±23.56	69.75±22.25
Concentration (Conc; 10^6/ml)	91.97±89.59	383.74±234.50	275.07±192.77	250.26±216.8
Percent normal sperm (PNS; %)	65.75±22.11	74.60±12.01	78.95±14.82	73.10±17.47
Head abnormalities (HA; %)	14.20±14.56	14.00±11.50	10.90±14.03	13.03±13.29
Total droplets (D; %)	8.60±9.93	3.90±3.67	5.80±6.49	6.10±7.31
Proximal droplets (PD; %)	6.80±9.11	2.35±2.25	2.9±3.73	4.02±6.07
Sperm chromatin structure assay (SCSA)				
HDS[1] (%)	4.18±4.96	3.02±1.50	2.39±2.04	3.19±3.25
DFI1[2] (%)	6.78±4.56	5.76±6.20	6.93±12.91	6.49±8.55
DFI2[2] (%)	11.40±6.98	12.07±12.67	11.53±15.97	11.67±12.23
Sperm-bos-halomax assay (SBH)				
Int[5] (%)	59.61±15.08	48.62±27.59	59.23±23.29	55.82±22.79
DC[4] (%)	21.35±10.54	21.50±10.01	21.40±9.32	21.41±9.80
Frag[3] (%)	19.05±10.61	29.88±26.12	19.38±18.25	22.77±19.72

[1]HDS: High DNA Stainability.

[2]DFI: DNA fragmentation index, measured by two alternative filters for red fluorescence (see methods).

[3]Frag: sperm with fragmented chromatin, exhibiting an evident halo.

[4]DC: sperm with signs of chromatin relaxation or decondencing chromatin.

[5]Int: intact sperm.

Table 2. Correlations between sperm chromatin fragmentation and traits measured at bull breeding soundness evaluation[1]

	Trait	Age	WT	BCS	FT	SC	Conc	MA	Mot	PNS	HA	PD	HDS	DFI 1	DFI 2	Int	DC
Across time	HDS	-0.22	-0.07	0.00	**-0.32**	-0.20	-0.17	**-0.30**	**-0.38**	-0.06	0.09	0.17					
	DFI1	0.02	-0.08	-0.16	-0.08	-0.20	-0.06	-0.08	-0.15	**-0.53**	**0.68**	0.16	0.18				
	DFI2	0.01	-0.08	-0.14	-0.07	-0.23	0.05	-0.06	-0.15	**-0.53**	**0.68**	0.13	0.08	**0.95**			
	Int	0.00	-0.09	-0.19	0.12	0.12	-0.25	-0.21	0.05	0.20	**-0.29**	0.00	-0.14	**-0.26**	**-0.28**		
	DC	-0.01	-0.01	0.05	-0.05	-0.11	-0.11	-0.07	-0.19	**-0.27**	**0.34**	0.20	**0.39**	0.22	0.20	**-0.51**	
	Frag	0.00	0.11	0.19	-0.12	-0.09	**0.35**	**0.28**	0.04	-0.10	0.17	-0.10	-0.03	0.19	0.23	**-0.90**	0.09
T1 (13 months)	HDS	0.04	0.24	-0.14	-0.41	-0.25	-0.32	**-0.52**	**-0.61**	0.04	0.06	0.08					
	DFI1	0.22	-0.01	**-0.60**	-0.41	-0.12	-0.17	-0.31	**-0.51**	**-0.55**	**0.57**	**0.56**	0.42				
	DFI2	0.18	-0.07	**-0.60**	-0.40	-0.10	-0.03	-0.27	**-0.45**	**-0.62**	**0.62**	**0.56**	0.24	**0.95**			
	Int	-0.18	-0.09	-0.04	0.33	0.09	0.05	-0.12	0.10	0.37	**-0.63**	-0.19	-0.44	**-0.49**	-0.40		
	DC	-0.05	-0.01	0.21	-0.26	-0.17	-0.06	-0.04	-0.21	-0.37	**0.47**	0.39	**0.55**	0.38	0.30	**-0.71**	
	Frag	0.31	0.14	-0.16	-0.21	0.04	-0.01	0.22	0.07	-0.16	0.43	-0.11	0.08	0.32	0.27	**-0.72**	0.02
T2 (18 months)	HDS	-0.27	0.11	-0.11	-0.06	0.34	-0.07	0.18	0.12	-0.16	0.17	0.07					
	DFI1	0.23	-0.14	-0.32	-0.03	-0.26	0.07	0.14	0.12	-0.42	**0.50**	-0.12	-0.23				
	DFI2	0.28	-0.15	-0.28	-0.07	-0.35	0.14	0.11	0.08	-0.40	**0.49**	-0.12	-0.28	**0.97**			
	Int	0.18	0.41	-0.17	0.35	0.15	-0.39	-0.22	0.26	0.25	-0.11	-0.05	-0.04	-0.15	-0.16		
	DC	-0.05	**-0.50**	0.01	-0.01	-0.06	-0.30	-0.17	-0.38	**-0.48**	0.42	0.15	0.45	0.09	0.10	-0.32	
	Frag	-0.18	-0.24	0.18	-0.37	-0.14	**0.53**	0.30	-0.13	-0.08	-0.05	0.00	-0.13	0.12	0.13	**-0.93**	-0.04
T3 (24 months)	HDS	0.32	-0.08	-0.40	**-0.49**	-0.32	0.00	0.06	-0.01	0.01	0.08	0.34					
	DFI1	0.03	-0.11	-0.07	-0.02	-0.25	-0.09	-0.12	-0.17	**-0.82**	**0.92**	0.05	0.30				
	DFI2	0.05	-0.13	-0.05	-0.04	-0.27	-0.01	-0.18	-0.18	**-0.83**	**0.94**	-0.02	0.21	**0.98**			
	Int	-0.02	-0.37	-0.26	0.09	0.25	0.04	-0.17	-0.16	0.20	-0.29	0.14	-0.01	-0.35	-0.38		
	DC	-0.22	0.41	0.02	0.02	-0.12	0.01	-0.02	-0.08	0.00	0.14	-0.13	0.16	0.28	0.27	**-0.68**	
	Frag	0.13	0.27	0.32	-0.12	-0.26	-0.05	0.23	0.25	-0.25	0.29	-0.12	-0.07	0.31	0.35	**-0.93**	0.36

[1]Age (age at bull breeding soundness evaluation), WT (liveweight), BCS (body condition score), FT (flight time), SC (scrotal circumference), MA (mass activity), Mot (percentage of cells with motility), PNS (percentage of normal sperm cells), HA (percentage of sperm cells presenting head abnormalities), PD (percentage of sperm cells presenting proximal droplets), HDS (high DNA stainability in SCSA), DFI (DNA fragmentation index, measured by two alternative filters, see methods in SCSA), DC (decondencing chromatin in sperm-bos-halomax Assay) and Frag (Fragmented in sperm-bos-halomax assay). **Significance: p value < 0.05 (bold); p value < 0.01 (bold and underlined).**

Supplementary files

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Title page:

Finding genes for economically important traits: Brahman cattle puberty

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Abstract. Age at puberty is an important component of reproductive performance in beef cattle production systems. Brahman cattle are typically late pubertal relative to *Bos taurus* cattle and so it is of economic relevance to select for early age at puberty. To assist selection and elucidate the genes underlying puberty, we performed a genome-wide association study (GWAS) using the BovineSNP50 chip (54,000 polymorphisms) in Brahman bulls (n = 1,115) and compared this to a previously performed GWAS of Brahman heifers (n = 843). The traits that defined age at puberty were age at which the first *corpus luteum* was detected (AGECL,

h^2 = 0.57 ± 0.12) in heifers and age at a scrotal circumference of 26 cm (AGE26, h^2 = 0.74 ± 0.04) in bulls. At puberty, heifers were on average older (751 ± 142 days) than bulls (555 ± 101 days), but AGECL and AGE26 were genetically correlated (r = 0.48 ± 0.09). There were 134 SNP associated with AGECL and 146 SNP associated with AGE26 (P < 0.0001). From these SNP, 32 (~ 22%) were associated (P < 0.0001) with both traits. These top 32 SNP were all located on chromosome BTA14, between 21.95 Mb and 28.4 Mb. These results suggest that the genes located in that region of BTA14 play a role in pubertal development in Brahman cattle. There are many annotated genes underlying this region of BTA14 and these are the subject of current research. Further, we identified a region on BTAX where markers were associated (P < 1.00E-8) with AGE26, but not to AGECL. Information about specific genes and markers add value to our understanding of puberty and potentially contribute to genomic selection. Therefore, identifying these genes contributing to genetic variation in AGECL and AGE26 can assist with the selection for early onset of puberty.

Key words: puberty, *corpus luteum*, scrotal circumference, *Bos indicus*, genome-wide association

Introduction

Age at puberty is an important component of cattle performance. It determines the beginning of reproductive life for any breeding animal and it influences generation interval, affecting the rate of genetic improvement and herd productivity. Selecting for early pubertal heifers and bulls is a practical approach for reducing the generation interval and potentially increasing fertility (Foster 1994; Johnston *et al.* 2010; Lesmeister *et al.* 1973; Siddiqui *et al.* 2008).

For genetic selection, puberty must be defined as a measurable and inherited trait. Puberty in heifers was defined by plasma progesterone concentration, ultrasound images of *corpus luteum* (CL) and detection of oestrous (Johnston *et al.* 2009; Romano *et al.* 2007; Shamay *et al.* 2005). Puberty in bulls was defined by scrotal circumference thresholds and was related to sperm concentration, motility and morphology (Bagu *et al.* 2004; Corbet *et al.* 2009; Lunstra *et al.* 1978; Siddiqui *et al.* 2008). Pubertal traits vary in heritability (h^2) from low to moderately high (Cammack *et al.* 2009).

There is a historical and increasing body of evidence reporting the genetic correlations between female and male puberty in cattle (Martin *et al.* 1992). Scrotal circumference of bulls correlates with puberty in their female relatives. Correlation estimates range from r = –0.71 to r = –0.15 (Burns *et al.* 2011). This evidence supports the indirect selection strategy for early pubertal heifers, using correlated traits measured in bulls. Given the size of the genetic correlation between these traits, some genes and genetic markers associated with puberty in heifers are likely to be associated to puberty in bulls and *vice versa*.

Bos indicus cattle, such as Brahman cattle, are reportedly older at puberty when compared to most *Bos taurus* breeds (Lopez *et al.* 2006; Lunstra and Cundiff 2003). It is of economic relevance to select for early age at puberty in Brahman cattle. To assist selection and elucidate the genes underlying fertility, we performed a genome-wide association study (GWAS) in a sample of bulls with a measurement for puberty: age at scrotal circumference of 26 cm (AGE26). This GWAS was compared to a previous GWAS performed on heifers measured for a puberty trait: age at the first *corpus luteum* (AGECL; Hawken, *et al.* 2011 submitted). The Brahman heifers measured for AGECL were the mothers of the bulls measured for AGE26. Heritability, genetic correlation and genes associated with AGECL and AGE26 are reported and discussed.

Material and Methods

Cattle and traits

Cattle were bred and supplied by the Cooperative Research Centre for Beef Genetic Technologies (Beef CRC) as described previously (Corbet *et al.* 2009; Johnston *et al.* 2009). In brief, data from 1,007 Brahman heifers and 1,118 Brahman bulls were included in the current analysis. The phenotypes of the heifers have been reported previously (Johnston et al., 2009; Hawken, *et al.* 2011 submitted). The bulls included in this experiment were the offspring of the heifers, which became breeding cows for the Beef CRC. Their pedigree consists of over 50 grandsire families: 54 bulls that sired heifers, which were mated to 55 industry sires to produce the bulls used in this study.

The traits that defined puberty were: age at which the first *corpus luteum* (CL) was detected (AGECL) in heifers and age at a scrotal circumference of 26 cm (AGE26) in bulls. The first CL was detected by ovarian ultrasound examinations conducted every 4 to 6 weeks, after heifers had reached an average liveweight of 200 kg (Johnston *et al.* 2009). AGECL was estimated from annotation of the date when the first CL was observed and the date of birth. Scrotal circumference (SC) was measured with a standard metal tape (Fordyce *et al.* 2006). Between weaning and 24 month of age, eight measurements of SC were taken for each bull, at 3 month intervals. Summary statistics for the age and SC of bulls at each of the eight time points are presented in Table 1. Using these repeated measurements for individual regressions, we interpolated the age when the bull achieved 26 cm of SC (AGE26, expressed in days). Achieving SC of 26 cm was considered a threshold for puberty in *Bos indicus* bulls. The threshold was different from the previously described 28 cm threshold because *Bos indicus* typically present a more elongated scrotum and smaller SC when compared to *Bos taurus* (Lunstra *et al.* 1988; McGowan *et al.* 2002; Silva *et al.* 2011). For a visual assessment of age versus SC in this population see Figure 1.

Genotypes

The Illumina BovineSNP50 array (Matukumalli *et al.* 2009; Van Tassell *et al.* 2008) was used to genotype samples according to the manufacturer's protocols (Illumina Inc., San Diego, CA). Positions for each SNP were based on the UMD3 assembly of the bovine genome sequence (available from http://www.livestockgenomics.csiro.au/cgi-bin/gbrowse/btauUMD3/). Genotypes for 843 heifers had been generated previously using the BovineSNP50 v1 array and were reported in a recent GWAS that analysed growth traits (Bolormaa *et al.* 2011).

In this study 1,118 bulls were genotyped using the Illumina BovineSNP50 v2 array. Quality control was similar to the previous study, with repeat samples included and the Bead Studio software (Illumina, 2006) used to determine the genotype calls. Animals with call rates less than 98% were excluded, resulting in 1,115 bulls being retained for analyses. SNP with auto-calling rates lower than 85% and SNP with a minor allele frequency (MAF) < 0.01 were excluded from further analyses.

For the current study, missing genotypes for bulls and cows were imputed using the BEAGLE 3.2 program (Browning and Browning 2010). These procedures yielded a total of 43,821 SNP used in the genome-wide association analysis. Genotype calls were coded as 0 for the homozygote of the first allele (A), 1 for the heterozygote, and 2 for the homozygote of the second allele (B). Alleles A and B were defined as per top/bottom rules from Illumina (http://www.illumina.com/documents/products/technotes/technote_topbot.pdf).

Genetic correlation and heritability

Genetic correlation and h^2 (for AGE26) were estimated fitting the two traits in a bivariate analysis using a mixed model and three generation pedigree relationships. For AGECL, fixed

125 effects included in the model and h^2 were reported previously (Johnston *et al.* 2009). Fixed
126 effects included in the model for AGE26 were contemporary group (bulls born in the same
127 year and location – or cohort – and post weaning location) and birth month. Solutions to the
128 effects in the model as well as variance components were estimated using VCE v.6 software
129 (Groeneveld and García-Cortés; http://vce.tzv.fal.de). Additionally, and for comparison
130 purposes, SNP-based estimates of genetic correlations were obtained from SNP effects across
131 the two traits, using the approach of Fortes et al. (2010).
132
133 ***Genome-wide association study***
134 Genome wide association studies (GWAS) were performed for AGECL and AGE26
135 separately. The effect of each SNP was estimated using the mixed model of Eq.1.
136 $$y_{ij} = X\beta + Zu + s_{jk} + e_{ij} \qquad [1]$$
137 where y_{ij} represents the vector of observations from the i-th cow at the j-th trait; X is the
138 incidence matrix relating fixed effects in β with observations in y_{ij}; Z is the incidence matrix
139 relating random additive polygenic effects in u with observations in y_{ij}; s_{jk} represents the
140 additive association of the k-th SNP on the j-th trait; and e_{ij} is the vector of random residual
141 effects. Fixed effects included in the model were the same as described for the models used to
142 estimate the genetic correlations and h^2. Tests for SNP association significance were
143 conducted using Qxpak5 (Perez-Enciso and Misztal 2004; Perez-Enciso and Misztal 2011).
144 Qxpak5 performs a likelihood ratio test, testing the model with versus model without the
145 SNP, against a chi-squared distribution with 1 degree of freedom and this was done one SNP
146 at a time. BTAX genotypes of males and females were not analysed together, as the traits
147 were analysed separately.
148 False discovery rates (FDR) were estimated using the equation of Eq.2.

$$FDR = \frac{nP}{k} \quad [2]$$

where n represents the total number of SNP included in the study (in the present study, n = 43,821); P is the P-value threshold being used and k is the actual number of associated SNP in the given *P-value* threshold.

The percentage of the genetic variance accounted by the i-th SNP was computed according to the formulae of Eq.3.

$$\%V_i = 100 \cdot \frac{2 p_i q_i a_i^2}{\sigma_g^2} \quad [3]$$

where p_i and q_i are the allele frequencies for the i-th SNP estimated across the entire population, a_i is the estimated additive effect of the i-th SNP on the trait in question (AGECL or AGE26), and σ_g^2 is the REML estimate of the (poly-)genetic variance for the trait in question.

Results

Descriptive statistics, h^2 and associated errors for AGECL and AGE26 are presented in Table 2. At puberty, heifers were on average older (751 ± 142 days) than bulls (555 ± 101 days). The heritability estimated for puberty in heifers was lower (AGECL, h^2 = 0.57 ± 0.12; Johnston *et al.* 2009) than for age at a scrotal circumference of 26 cm (AGE26, h^2 = 0.74 ± 0.04) in bulls.

The traits AGECL and AGE26 were genetically correlated with r = 0. 48 ± 0.09 estimated using REML methods. The SNP-based estimate of the genetic correlation gave similar values to the REML estimate, when using the subset of 505 SNP with $P < 0.05$ for both AGECL and AGE26. SNP-based correlations using a more stringent P-value were based in a small proportion of the total SNP and yield higher correlations (Figure 2).

Figure 3 provides an overview of GWAS results presented as Manhattan plots (i.e., significance on the y-axis versus genome map position on the x-axis) for AGE26 and AGECL. Two regions showed clear association peaks in the GWAS of AGE26, one on the BTAX chromosome and another on BTA14. The GWAS of AGECL also shows a peak on BTA14 for the same region where SNP were significant for AGE26. There were 134 SNP associated with AGECL and 146 SNP associated to AGE26 ($P < 0.0001$, FDR = 0.03). Of these SNP, 32 (~ 22%) were associated with both traits ($P < 0.0001$, Table 3). These 32 SNP that were in common for both traits, were all located on BTA14, between 21.95 Mb and 28.4 Mb. The most significant SNP for AGECL was Hapmap23509-BTC-073113 at position 27,198,715 of BTA14 (P = 1.36E-09). The most significant SNP for AGE26 was BTA-30242-no-rs at position 85,495,447 of chromosome X (P = 5.35E-13). The effects, P-values and proportion of genetic variance explained for each of the 32 SNP associated ($P < 0.0001$) with both AGECL and AGE26 are presented in Table 4. The effects, P-values and proportion of the variance explained for the SNP underlying the association peak located on BTAX are also reported (Table 5).

The nearest annotated genes and their distance to the reported SNP were determined using the UMD3.0 bovine genome assembly (Tables 4 and 5). However, to consider only the genes nearest to each SNP could be misleading. It is preferable to consider all genes mapped to the association regions. In total, 23 genes mapped to the association region in chromosome 14: *ATP6V1H, CA8, CHD7, CSF2RA, FAM110B, IMPAD1, NPBWR1, NSMAF, OPRK1, PCMTD1, PENK, POLR2K, RAB2A, RB1CC1, RGS20, RLBP1L1, RP1, SDR16C6, SNTG1, SOX17, TGS1, TOX* and *XKR4*. Also, 46 genes mapped to the association region in chromosome X: *ABCB7, AKAP4, APOL, AR, ARR3, BRWD3, CACNA1F, CCDC120, CHIC1, CHM, CLCN5, CXCR3, CYLC1, DGAT2L3, DGAT2L6, EDA, EFNB1, FGF16, FOXP3, GPKOW, H11CXORF26, KIF4A, LRCH2, MGC140080, MGC152340, MIR374A,*

MIR374B, NHSL2, PAGE4, PJA1, PLS3, POU3F4, RBM10, SLC9A7, SNX12, STARD8, SUV39H1, TAF1, TAF9B, TIMM17B, TMEM28, UPRT, USP11, USP27X, ZDHHC15 and *ZNF182*.

Discussion

Heritability estimates of cattle puberty in the literature are variable and influenced by population, breed, management and environmental factors, as well as the use of different phenotypic measurements (Cammack *et al.* 2009; Martin *et al.* 1992). Heritability for age at puberty in the Beef CRC Brahman heifers (AGECL, $h^2 = 0.57$) was reported previously (Johnston *et al.* 2009) and it is within the range, from 0.20 to 0.67, of other estimates for age at puberty (Arije and Wiltbank 1971; Laster *et al.* 1979; Smith *et al.* 1976). Reported heritability estimates for scrotal circumference range from 0.39 to 0.75 (Corbet *et al.* 2009; Lunstra *et al.* 1988; Martinez-Velazquez *et al.* 2003). Our result for puberty in bulls (AGE26) was closer to the higher end of that range ($h^2 = 0.74$). Overall and in agreement with our results, the heritability of puberty in cattle ranges from moderate to high, allowing for improvement through genetic selection.

Pubertal traits measured in heifers (AGECL) and bulls (AGE26) had a positive genetic correlation. This result confirms previous reports that showed bigger scrotal circumference in bulls were correlated with early puberty in their female relatives (Burns *et al.* 2011; Martin *et al.* 1992). Previous evidence and our results point to the practicality of selecting for early pubertal cattle since both heifers and bulls can be selected with beneficial correlated effects.

Considering the genetic correlation, genes and SNP associated with puberty in heifers were likely to be relevant for puberty in bulls and vice-versa. The possibility to estimate genetic correlations from SNP effects was previously contemplated by Fortes et al. (2010). In

the present study, we established that the 505 SNP found to be associated with puberty in both bulls and heifers at $P < 0.05$ would give rise to an estimated genetic correlation of 0.48 which is in close agreement with the pedigree-based REML estimate of 0.48. However, further pruning of SNP on joint significance across both traits resulted in an inflated estimated of SNP-based correlation (Figure 2). This overestimation was largely attributed to the associations on BTA14, where a large number of SNP associated with puberty were identified in both bulls and heifers. These SNP located on BTA14 are located close to *RP1*, *XKR4* and *TOX*. These genes are not known to affect puberty in any species. However, the region on BTA14 includes another 20 annotated genes and presents homology to human chromosome HSA8q12, which was associated with height (Gudbjartsson *et al.* 2008). Among those genes, *PENK, RPS20, SNORD54* and *PLAG1* are plausible functional candidates. *PENK* has a role in *GnRH* regulation (Rosie *et al.* 1992; Taylor *et al.* 2007). *RPS20* and a small RNA (*SNORD54*) were recently associated with calving ease (Pausch *et al.* 2011). The alleles that lowered calving-ease had a positive effect on growth traits (Pausch *et al.* 2011). Calving ease is a trait influenced by the size of the calf and so, similarly to stature, it reflects frame size. Cattle with smaller frame size achieve puberty earlier than those with larger frame size (Vargas *et al.* 1999). Taken together this evidence could point to *RPS20* and *SNORD54* as pleiotropic genes affecting puberty and calving-ease mainly through their effect on growth. Another recent study argued that *PLAG1* was the relevant gene underlying this region on BTA14 and affecting bovine stature (Karim *et al.* 2011). Considering the physiological link between growth and puberty, it is possible to hypothesize that the association region in chromosome 14 may relate to a gene with many pleiotropic effects or a functional polymorphism that affects more than one gene. There is already evidence for a functional SNP in this region affecting the expression levels of multiple genes (Karim *et al.* 2011).

The two most significant SNP for AGE26 were located on BTAX and not on BTA14. The genes nearest to the most significant SNP, *EDA* and *DGAT2L3,* do not appear to play a role in reproduction given the current evidence from literature. A formal discussion of the functional link between these genes and cattle puberty is beyond our present objective. However, the association peak on BTAX does include three genes that are positional and functional candidate genes: *AR* (androgen receptor), *TAF1,* and *TAF9B* (both TATA box binding protein (TBP)-associated factors). These genes are candidate genes for the following reasons. The androgen receptor is known to play a role in sexual development, specifically, it affects spermatogenesis, testis localization and testis size in mice models (Verhoeven *et al.* 2010). In pigs, the *AR* is considered a candidate gene for reproduction and performance traits (Trakooljul *et al.* 2004). Manipulation of fetal androgen exposure alters the timing of puberty in sheep (Jackson *et al.* 2008). The genes *TAF1* and *TAF9B* encode transcription factors that form the TFIID complex, a regulator of cell cycle and differentiation. Testis specific *TAF* proteins were reported as relevant for spermatid differentiation in *Drosophila* (Hiller *et al.* 2004). Taken together this evidence and our results support *AR*, *TAF1* and *TAF9B* as candidate genes for scrotal development and puberty in Brahman bulls.

Results of this study are consistent with the hypothesis that the genes underlying the associated regions on BTA14 and BTAX play roles on defining age at puberty in Brahman cattle. Candidate genes mapping to these regions are the subject of ongoing research. Information about specific genes and markers will add value to genomic selection. Therefore, identifying these associated regions contributing to genetic variation in AGECL and AGE26 should assist with the selection for early onset of puberty in Brahman cattle.

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448

449

450

451 **Tables**

452 **Table 1. Means ± Standard Errors for measurements of scrotal circumference (SC) at 8**

453 **time points.**

Time Point	Age	SC
1	182.30 ± 133.64	17.024 ± 0.86
2	256.43 ± 209.77	18.03 ± 0.93
3	313.22 ± 240.97	19.68 ± 1.29
4	373.73 ± 312.85	21.23 ± 1.72
5	443.66 ± 359.56	24.55 ± 2.34
6	525.52 ± 443.19	26.38 ± 2.44
7	617.87 ± 486.12	28.03 ± 2.50
8	702.78 ± 539.66	29.88 ± 2.71

454

455 **Table 2. Summary statistics and heritability estimates for AGECL and AGE26.**

	AGECL**	AGE26
Definition	Age at the first *corpus luteum*, in days	Age at the scrotal circumference of 26 cm, days
N animals	1,007	1,118
Mean	750.60	555.06
Std.*	142.14	100.52
Min	394.00	284.00
Max	1,211.00	1,174.00
h^2 ± SE	0.57 ± 0.12	0.74 ± 0.04

456 ***Std.: Standard deviation; **Phenotype previously reported (Johnston *et al.* 2009).

457 **Table 3. Number of SNP associated with age at the first *corpus luteum* (AGECL), age at**

458 **the scrotal circumference of 26 cm AGE26 and both traits at various *P*-value thresholds**

***P*-values**	**AGECL**		**AGE26**		**Both**
	SNP*	**FDR****	**SNP**	**FDR**	
$P < 0.05$	4924	0.44	3865	0.57	505
$P < 0.01$	1629	0.27	1241	0.35	119
$P < 0.001$	410	0.11	315	0.14	49
$P < 0.0001$	134	0.03	146	0.03	32
$P < 0.00001$	61	0.01	74	0.01	9

459 ***SNP: number of single nucleotide polymorphisms associated

**FDR: false discovery rate

Table 4. Effect, *P*-values and proportion of the variance explained for SNP associated (*P* < 0.0001) with both AGECL and AGE26 and located in CHROMOSOME 14.

	Chr. 14		AGECL			AGE26				
SNP	**Position**	**MAF**	**Effect**	***P***	**%Var.**	**Effect**	***P***	**%Var.**	**Gene**	**Distance**
Hapmap27177-BTA-147405	21,954,567	0.40	24.52	9.83E-06	8.22	16.86	1.79E-05	2.17	CSF2RA	62,207
Hapmap32552-BTA-129045	22,323,719	0.42	27.89	8.00E-07	10.79	16.80	1.66E-05	2.19	SNTG1	0
BTB-01252028	22,544,496	0.45	-28.34	4.10E-07	11.34	-16.48	4.71E-05	2.14	PCMTD1	124,865
ARS-BFGL-BAC-12159	22,587,081	0.50	-27.55	4.96E-07	10.80	-16.97	2.47E-05	2.29	PCMTD1	82,280
Hapmap31202-BTA-162588	23,988,778	0.44	-27.01	1.20E-06	10.25	-16.74	2.96E-05	2.20	RP1	1,415
ARS-BFGL-NGS-104268	**24,057,354**	**0.42**	**-28.3**	**1.20E-07**	**11.16**	**-25.00**	**8.45E-10**	**4.86**	RP1	58,016
BTB-01532239	24,437,778	0.48	-29.43	3.24E-08	12.31	-15.86	6.30E-05	2.00	XKR4	0
BTB-01530778	24,482,969	0.45	-27.06	2.57E-07	10.33	-16.39	3.96E-05	2.12	XKR4	0
BTB-01530788	24,524,205	0.48	-29.33	4.13E-08	12.23	-15.61	8.21E-05	1.94	XKR4	0
BTB-01530836	24,573,257	0.48	29.33	4.13E-08	12.23	15.89	6.10E-05	2.00	XKR4	0
BTB-00557532	24,643,266	0.49	-29.58	3.32E-08	12.45	-15.91	5.95E-05	2.01	XKR4	32,308
Hapmap41234-BTA-34285	25,107,556	0.47	-30.12	1.45E-08	12.85	-17.69	4.39E-06	2.48	LOC526726	0
BTB-02056709	25,175,950	0.48	28.47	5.51E-08	11.53	18.10	4.72E-06	2.60	SDR16C6	0
Hapmap46986-BTA-34282	25,307,116	0.50	-28.40	5.09E-08	11.48	-18.73	1.19E-06	2.79	PENK	84,125
BTB-01779799	25,351,733	0.44	-28.87	1.01E-07	11.71	-16.15	3.01E-05	2.05	PENK	128,742
ARS-BFGL-NGS-529	25,638,580	0.44	-29.06	1.04E-07	11.82	-17.98	3.65E-06	2.53	IMPAD1	77,701
ARS-BFGL-NGS-111395	25,731,992	0.48	28.35	8.71E-08	11.43	16.83	1.65E-05	2.25	IMPAD1	171,113
BTA-97369-no-rs	25,887,784	0.44	-24.14	5.73E-06	8.16	-16.06	7.68E-05	2.02	FAM110B	162,458
Hapmap24966-BTC-054594	26,080,801	0.49	-29.67	6.43E-09	12.52	-15.76	9.54E-05	1.97	FAM110B	0
Hapmap32434-BTC-011497	26,473,490	0.40	-27.53	2.09E-07	10.39	-17.06	2.49E-05	2.23	NSMAF	0
ARS-BFGL-NGS-35159	26,508,236	0.41	27.97	1.27E-07	10.78	16.54	4.41E-05	2.11	NSMAF	12,525
Hapmap28828-BTC-011250	26,713,734	0.40	-27.19	3.70E-07	10.08	-16.97	2.90E-05	2.19	TOX	0
Hapmap30932-BTC-011225	26,766,010	0.40	-26.69	5.31E-07	9.71	-16.60	4.25E-05	2.10	TOX	0
Hapmap25761-BTC-065280	26,949,215	0.44	26.78	1.96E-07	10.07	18.53	2.32E-06	2.69	TOX	7,489
BTB-01280026	27,035,971	0.40	27.28	2.63E-07	10.17	17.02	2.73E-05	2.21	TOX	94,245
Hapmap27934-BTC-065223	27,155,254	0.40	27.69	1.84E-07	10.47	16.31	5.58E-05	2.03	TOX	213,528
Hapmap23509-BTC-073113	**27,198,715**	**0.46**	**-31.20**	**1.36E-09**	**13.79**	**-17.80**	**4.32E-06**	**2.49**	TOX	256,989
Hapmap23060-BTC-072978	27,360,366	0.40	29.11	4.22E-08	11.54	21.53	1.95E-07	3.53	CA8	277,183
Hapmap26621-BTC-072953	27,380,992	0.39	-28.75	8.60E-08	11.16	-20.56	6.53E-07	3.19	CA8	256,557
BTB-00560182	28,247,205	0.37	24.69	4.87E-06	8.08	17.28	3.96E-05	2.21	CHD7	75,924
BTB-02008412	28,300,924	0.43	-22.06	1.96E-05	6.79	-16.29	7.73E-05	2.07	CHD7	129,643
BTB-00561430	28,443,928	0.38	-28.39	3.36E-07	10.85	-16.35	7.13E-05	2.01	RLBP1L1	94,261

SNP positions and distance to nearest gene are reported in base pairs and based on the UMD3 assembly of the bovine genome sequence. Most significant SNP in terms of *P-values* are reported in bold.

Table 5. Effect, *P*-values and proportion of the variance explained for SNP underlying the association peak in the X chromosome observed for AGE26.

	Chr. X		AGECL			AGE26				
SNP	**Position**	**MAF**	**Effect**	***P***	**%Var.**	**Effect**	***P***	**%Var.**	**Gene**	**Distance**
Hapmap32855-BTA-155265	69,196,959	0.18	0.65	9.32E-01	0.00	19.85	4.96E-09	1.84	LOC518911	136,499
Hapmap27896-BTA-26538	70,057,309	0.18	0.65	9.32E-01	0.00	19.85	4.96E-09	1.84	CYLC1	109,833
Hapmap32639-BTA-158837	70,234,032	0.18	2.32	7.65E-01	0.04	19.88	5.92E-09	1.82	LOC782514	122,078
Hapmap24863-BTA-154164	71,950,688	0.18	0.65	9.32E-01	0.00	20.23	3.18E-09	1.90	PLS3	144,154
Hapmap24353-BTA-19502	72,447,651	0.18	-0.65	9.32E-01	0.00	-20.23	3.18E-09	1.90	LOC784663	268,945
Hapmap31832-BTA-160852	73,379,138	0.18	0.57	9.40E-01	0.00	20.14	3.31E-09	1.88	POU3F4	153,386
Hapmap25915-BTA-161991	73,483,564	0.18	1.08	8.89E-01	0.01	20.14	3.31E-09	1.87	POU3F4	257,812
Hapmap26716-BTA-150917	75,234,781	0.18	0.65	9.32E-01	0.00	19.85	4.96E-09	1.84	APOL	0
Hapmap32363-BTA-155726	75,506,946	0.18	-0.65	9.32E-01	0.00	-19.85	4.96E-09	1.84	APOL	241,844
Hapmap31156-BTA-153166	77,369,290	0.18	0.57	9.40E-01	0.00	20.14	3.31E-09	1.88	LOC523251	599,379
Hapmap25932-BTA-30257	81,477,451	0.18	4.97	5.21E-01	0.21	21.17	3.69E-10	2.08	LOC512493	0
Hapmap60788-rs29017234	82,408,727	0.15	10.70	1.88E-01	0.85	22.25	7.91E-10	2.05	CHIC1	6,068
Hapmap49114-BTA-30310	84,335,452	0.15	6.76	4.18E-01	0.34	-22.72	2.61E-10	2.13	CXCR3	19,873
Hapmap24012-BTA-158546	84,867,168	0.18	-4.37	5.52E-01	0.16	23.83	2.22E-12	2.66	SNX12	6,393
Hapmap32396-BTA-164240	85,334,862	0.18	-3.54	6.39E-01	0.10	24.12	1.12E-12	2.69	KIF4A	0
ARS-BFGL-NGS-98019	85,463,982	0.17	-3.01	6.96E-01	0.07	24.03	2.24E-12	2.61	ARR3	0
BTA-30242-no-rs	**85,495,447**	**0.17**	**-5.51**	**4.67E-01**	**0.25**	**24.53**	**5.35E-13**	**2.76**	DGAT2L3	4,439
Hapmap49950-BTA-30244	85,589,749	0.13	-13.33	1.09E-01	1.13	22.56	4.15E-09	1.81	DGAT2L6	12,013
Hapmap33084-BTA-164247	**85,833,472**	**0.17**	**-4.61**	**5.43E-01**	**0.17**	**24.51**	**6.76E-13**	**2.74**	EDA	0
Hapmap52858-rs29015876	86,556,922	0.14	-12.47	1.43E-01	1.07	24.45	4.22E-11	2.30	PJA1	47,130
Hapmap39370-BTA-108497	91,270,661	0.10	-16.49	1.10E-01	1.35	30.30	2.32E-11	2.55	LOC100141016	0
Hapmap47906-BTA-29386	93,631,688	0.14	-5.80	4.52E-01	0.23	21.70	2.71E-09	1.81	LOC784528	106,632

SNP positions and distance to nearest gene are reported in base pairs and based on the UMD3 assembly of the bovine genome sequence. Most significant SNP in terms of *P-values* are reported in bold.

Legend to figures

Figure 1. Age and Scrotal Circumference (SC) measured across 8 time points. Scatter plot of the age in days (x-axis) versus the SC in cm (y-axis), with time points represented by alternating colours (black and white).

Figure 2. Genetic and SNP-based correlations between AGE26 and AGECL. AGE26 is the age when a scrotal circumference of 26 cm was achieved and AGECL is the age at the first *corpus luteum*, both expressed in days. Genetic correlation was estimated via REML and is represented by the red diamond (r = 0.480). SNP-based correlations were estimated using increasingly more stringent *P*-values (in the x-axis) that yielded smaller number of SNP (gray triangles) and higher estimated SNP-based correlations (gray squares). Both the percentage of SNP used and the resulting correlation are expressed in the y-axis (from 0 to 1).

Figure 3. Manhattan plots for AGE26 and AGECL. Plots represent the associations between genome-wide SNP and age at a scrotal circumference of 26 cm (AGE26), as well as, SNP and age at the first *corpus luteum* (AGECL). In both plots chromosomal positions are in the x-axis and –Log (*P*-values) are in the y-axis.

Figures

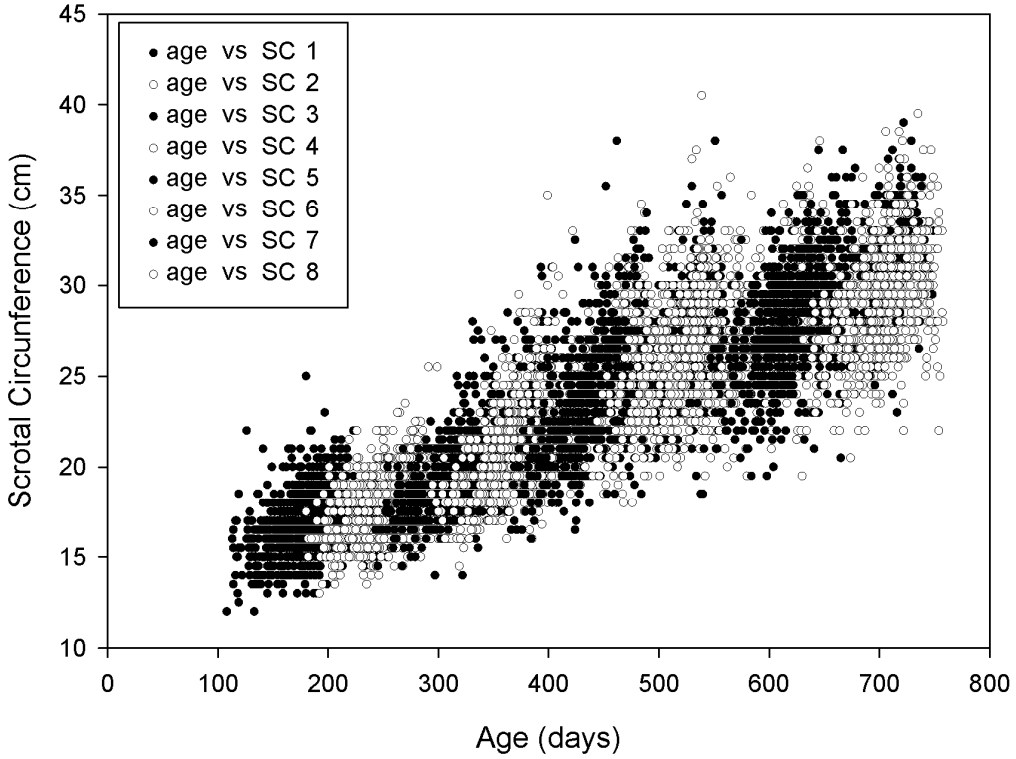

Figure 1. Age and Scrotal Circumference (SC) measured across 8 time points.

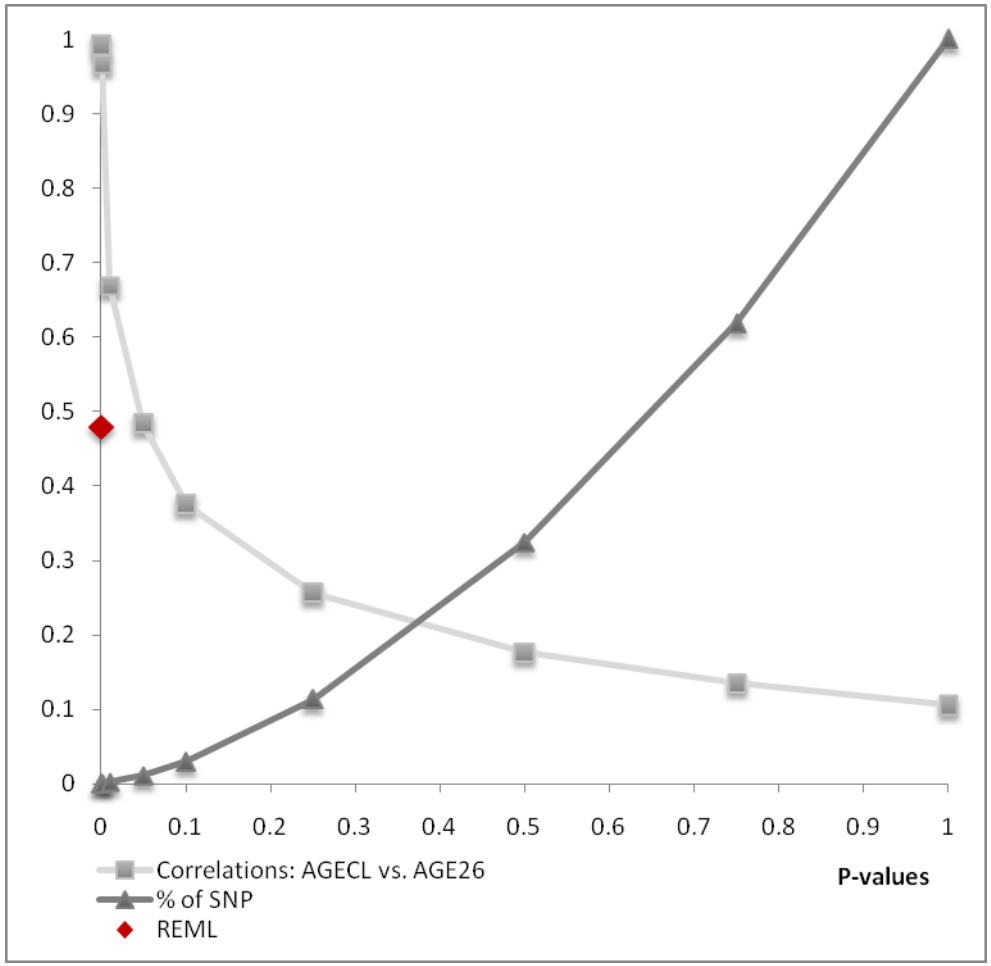

Figure 2. Genetic and SNP-based correlations between AGE26 and AGECL.

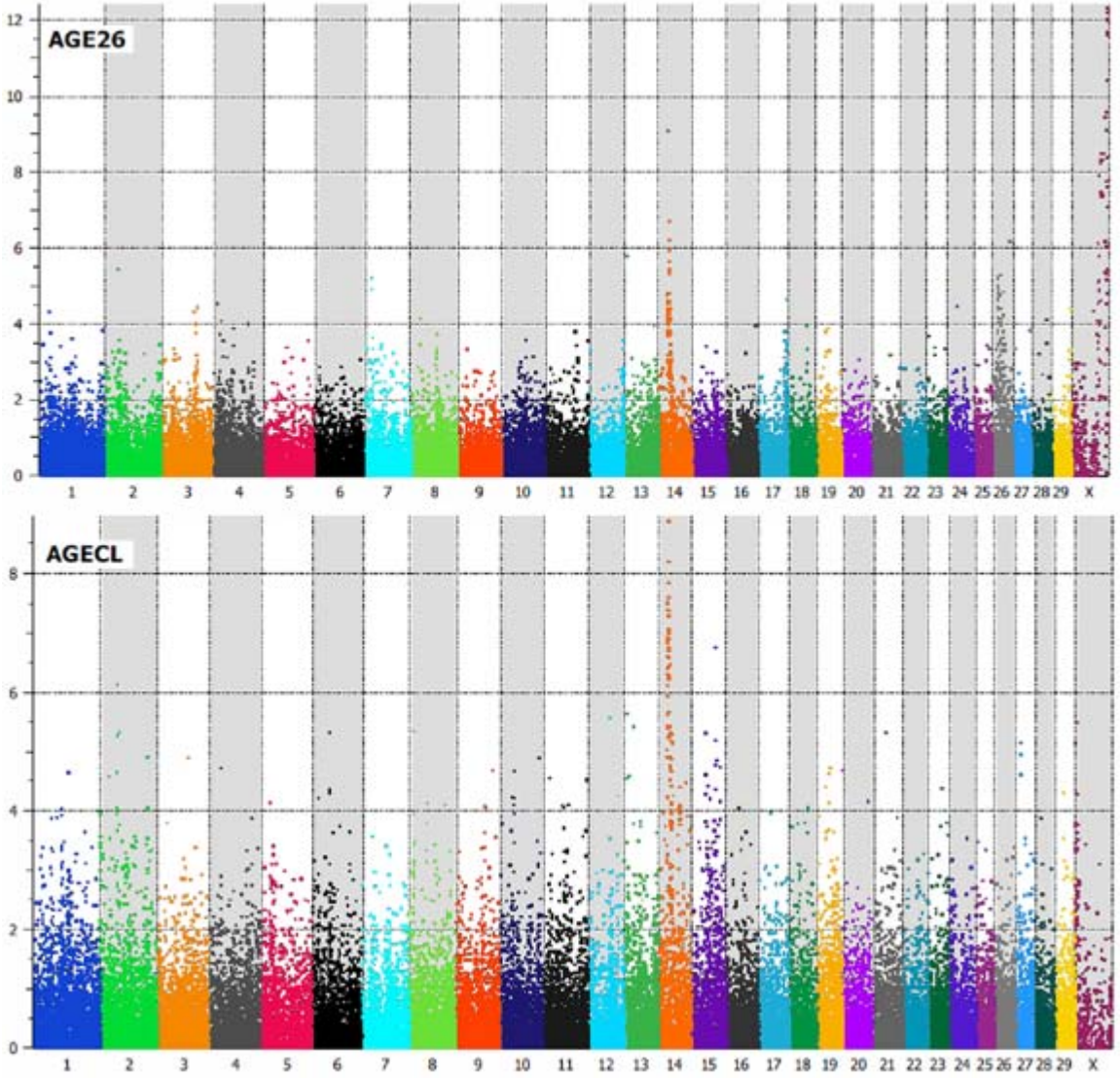

Figure 3. Manhattan plots for AGE26 and AGECL.