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Prepared by: Marina R. S. Fortes[1], Sigrid A. Lehnert[2] and Matthew Kelly[1]

[1]The University of Queensland and [2]CSIRO, Agricultural Flagship

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Ideal markers for tropically adapted cattle - proof of concept: functional mutations for bull fertility

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Abstract

This project was a 'proof of concept' for identifying functional mutations via a combination of genome-wide association studies (GWAS) and sequenced genomes. Targeting 69 sequenced genomes enabled exploration of genes identified via GWAS (during the Beef CRC) with greater detail, leading to the discovery of 13 putative functional mutations. These mutations are new genetic markers and 10 of them were significantly associated with scrotal circumference and percentage of normal sperm in an independent population of mixed-breed beef cattle. These associations pointed to 9 genes in the X chromosome and one key gene in chromosome 5. The key genes in X were *LOC100138021, CENPI, TAF7L, NXF2, CYLC1, UXT, SPACA5, TEX11,* and *AR*. The gene *HELB*, mapped to chromosome 5, was associated to scrotal circumference. We also confirmed the association between the *PLAG1* gene mutation and scrotal circumference. Such validation of markers justified further exploitation of the mixed-breed population. Subsequently, 1,012 bulls of this population were genotyped using the 74,000 SNP chip. Chip genotyping enabled new genome-wide association studies and genomic selection analyses. All genotypes were incorporated to the Beef CRC legacy database, to support continued improvement of genomic selection strategies for tropical beef cattle. In our analysis, incorporating these new 1,012 bulls in the training dataset improved the genomic selection accuracy (1 to 4% higher accuracies) for scrotal circumference measurements across populations and breeds. The inclusion of the functional mutations had a significant ($P < 0.001$) and positive impact in the genomic prediction models for scrotal circumference. Functional mutations are likely to have similarly significant impact in other traits and can be incorporated in genomic models once they are discovered. Further research is required to discover more functional mutations related to female reproductive traits and production traits (i.e. meat traits or adaptation traits).

Executive summary

The need to improve prediction of reproductive phenotypes in tropical beef cattle populations was the motivation for the research performed and presented herein. Improved predictions will support selective breeding for cattle with higher genetic merit for reproductive performance. Genetic improvement of reproductive performance is possible since phenotypes related to fertility, such as scrotal circumference (SC), percentage of normal sperm (PNS), age at puberty (AGECL) and post-partum anoestrus interval (PPAI) are heritable. In Brahmans for example, the heritability estimated for SC was 0.75, for PNS was 0.15, for AGECL was 0.57 and for PPAI was 0.52 (Corbet et al., 2009; Johnston et al., 2009; Johnston et al., 2010; Corbet et al., 2011; Corbet et al., 2013; Johnston et al., 2013a). Heritable phenotypes are influenced by genes and mutations. Mutations that affect, or "cause", the phenotype do so because of their molecular function. Mutations with molecular function, termed functional mutations, are those that for example change the amino acid sequence of the protein coded by the gene. Identification of functional mutations associated with reproductive phenotypes was the objective of this research project, because functional mutations are considered ideal gene markers. Functional mutations are more likely to be associated to phenotypes across breeds and time reducing the need for re-calibration of genomic predictions. The practical use of these ideal markers is to aid selective cattle breeding. The aim is to produce more accurate breeding values.

The reproductive phenotypes targeted in the current research were important traits related to bull fertility: SC and PNS. Up to 4% higher accuracies in the prediction of estimated breeding values (EBVs) were achieved with the use of DNA technology and genomic selection, as demonstrated in our results. Specifically, we have demonstrated the ability to identify functional mutations in key genes via combination of genome-wide association studies (GWAS) and sequenced genomes, leading to the discovery of 13 putative functional mutations. These mutations are new genetic markers and 10 of them were significantly associated with SC and PNS in an independent population of mixed-breed beef cattle. These functional mutations can form the basis for improved EBV predictions for scrotal circumference, because they had a significant impact when added to pedigree and genomic models ($P < 0.001$). Functional mutations explained additional genetic variance for scrotal circumference (Table 1). A similar improvement and significance was not observed for PNS, as the effect and association of the mutations was primarily to SC. This was a proof of concept project that used available validation animals from the Beef CRC legacy database, which were not included in the initial GWAS analysis. Now that the concept is proven and we have demonstrated the ability to identify functional mutations, this approach can be expanded to other phenotypes, including traits related to female fertility. Improvement of female fertility is a recognised industry priority. To improve female fertility, the identification of functional mutations associated with AGECL and PPAI could assist selective breeding. From the current project one functional mutation was associated to female fertility, mapped to the *HELB* gene.

Table 1. Percentage of variance explained by 11 functional mutations (FM) independently or as part of a pedigree or genomic model.

Model*	**SC12**	**SC18**	**SC24**	**PNS24**
FM only	**5.46**	**5.32**	**5.51**	0.07
FM + A	**5.17**	**4.16**	**4.62**	0.22
FM + GRM	**4.39**	**3.51**	**4.37**	0.19
FM + GRM-X	***1.74***	0.39	***1.17***	0.00

*FM only: model included only functional mutations (11 markers) and fixed effects; FM + A: model included a pedigree matrix, functional mutations and fixed effects; FM + GRM: model included genomic relationship matrix from autosomes (no chromosome X), functional mutations and fixed effects; FM + GRM-X model included genomic relationship matrix from autosomes and chromosome X, functional mutations and fixed effects. In most models for prediction of scrotal circumference, the addition of the functional mutations had significant impact ($P < 0.001$, for the results highlighted in bold and $P < 0.05$ for results in bold and italic). The genetic variance explained by these mutations was higher for models that did not include the X chromosome, which are commonly used in current EBV predictions.

Genome-wide data and key genes genotyped for 1,012 bulls measured for SC, PNS and a range of reproductive traits were created in this project and incorporated in the Beef CRC legacy database. This new genomic resource can be used for further research and to improve current delivery of estimated breeding values for SC, PNS and other genetically correlated traits. Currently, an EBV for SC is available through Breedplan, but an EBV for PNS is not. We have estimated EBVs for PNS for bulls genotyped in three MLA projects: **B.NBP.0604** (1,115 Brahman bulls), **B.NBP.0723** (1,019 Tropical Composite bulls) and **B.NBP.0786** (current project, 1,012 mixed-breed bulls). Our data indicates that it is possible to estimate EBVs for PNS, which could be an additional selection tool for bull fertility. In the previous bull power project, PNS was the trait with higher correlation to calf-getting ability (Holroyd et al., 2002). Further, female phenotypes such as AGECL and PPAI are genetically correlated to SC and PNS (Johnston et al., 2013b). The resource created in this project will contribute to selective breeding to improve male and female reproductive traits.

Industry can benefit from this work in two timeframes: 1) Immediate use of the genotype resources created in this project is ongoing by incorporation of data in the prediction equations derived by AGBU and accessible through Breedplan, and 2) In the near future, functional mutations discovered herein could be part of a focused SNP panel delivered as a cost-effective alternative to current genomic selection. Breeders of tropical composite and *Bos indicus* infused cattle will benefit from the specific knowledge and data generated in this project. Improvement of DNA tests tailor-made for tropical breeds is necessary to encourage technology adoption. The mutations reported herein are a step towards improved DNA tests for tropically adapted breeds.

Table of contents

1 Background

This project was a 'proof of concept' for identifying functional mutations via a combination of genome-wide association studies (GWAS) and sequenced genomes. The CRC for Beef Genetic Technologies (Beef CRC) conducted GWAS for female and male reproductive traits in Brahman and Tropical Composite cattle (Fortes et al., 2012a; Fortes et al., 2012b; Hawken et al., 2012; Fortes et al., 2013b). The genotypes generated on these GWAS also formed the basis for genomically-enhanced EBV (gEBV) for female and male reproductive traits (Zhang et al., 2013; Barwick et al., 2014). These gEBV have accuracies of ~40% when applied to a breed represented in the Beef CRC reference population (e.g. Brahman). Across breed accuracies for EBVs or when genomic predictions are applied to a breed which was not included in the reference population tend to be lower than within-breed predictions, as shown using meat quality and production traits (Boerner et al., 2014). The genomic predictions developed using the Beef CRC data can contribute to the implementation of gEBVs in Australian beef cattle breeding (Boerner et al., 2014), although these across breed predictions are generally lower than reported within breed-predictions.

In order to increase the accuracy of gEBV when applied across breeds, the inclusion of functional mutations in the genotypic data used for the prediction is of value (Kemper et al., 2015). The current project therefore was set out to identify and validate putative functional mutations for cattle reproductive traits. Ideally, functional mutations would be identified for female reproductive traits, given their relevance to the industry (Burns et al., 2010). Currently, no suitable validation dataset is available for female traits measured during the Beef CRC. Instead, two bull reproductive traits - scrotal circumference (SC) and percentage of normal sperm (PNS) - were used to test and prove the approach. An existing validation dataset from the Beef CRC was employed for this purpose.

The dataset used in this project was generated during the Beef CRC, but consists of the SC and PNS phenotypes of 1,012 bulls that had never been used in any of the previous GWAS analyses. These additional bulls are Brahman (n = 113), Tropical Composites (n = 741) and crosses between the two breeds (n = 167). This population represents an ideal validation resource for genetic markers discovered previously either in Brahman or in Tropical Composites (Fortes et al., 2012a; Fortes et al., 2013b). The bulls were raised as contemporaries of those previously studied in the Beef CRC and were measured in the same environments, using the same methodology.

Previous GWAS (Fortes et al., 2012a; Fortes et al., 2013b) provided information about the location of hotspots on the genome associated with the target phenotypes and accelerated the process for identification of functional mutations. The profound breed differences between Brahman and Tropical Composites detected in previous GWAS have important implications for the Australian beef industry and the multiple breeds it employs. The development and application of DNA-based predictions to the northern beef industry needs to take into account the genomic architecture of the trait for each breed and use to its advantage in situations when diverse breeds yield similar results. Similar results were detected for chromosomes X and 14 for reproductive traits in males (see final report B.NBP.0723). The aim of the project was to look for functional mutations near the top gene markers that are currently known, with a special focus on chromosome X, because this is the most likely location for functional mutations associated with male reproductive traits. The practical outcomes of identifying functional mutations remains very promising as these could be the logical basis of a reduced and cost-effective marker panel for

fertility selection in northern Australia. Further, functional mutations are more likely to work across breeds and help sustain multi-breed predictions.

Beyond the 'proof of concept' objective, investigating the genetics of male reproductive traits has merit on its own. In livestock breeding, sires have an important effect in disseminating superior genetic merit, particularly in situations where artificial insemination (AI) is used (Edwards et al., 2015). Sires with better fertility guarantee the efficiency dissemination of superior genetic merit for any trait under selection (i.e. meat quality or parasite resistance). Breeding sires for fertility requires the measurement of reproductive traits, such as SC and PNS that serve as indicators of fertility. Selection for SC and PNS traits is important because sires with superior fertility also contribute to overall conception rates and reproductive performance of the herd. Conception rates have economic impact in beef production systems (Wolfova et al., 2005). Improved conception rates as well as effective dissemination of superior genetics would increase the economic return of beef production systems. Due to cost, male reproductive traits other than SC are not commonly measured and evaluated in animal breeding programs. The identification of functional mutations associated with SC and PNS could assist animal breeding, via genomic selection. The outcome would be bulls with improved fertility.

An indirect benefit of improved bull fertility could be cow fertility, given the genetic correlations between male and female reproductive traits. For example, SC measured at 12 months and PNS measured at 24 months of age have a moderate and negative (favourable) genetic correlation with female puberty and a moderate and positive genetic correlation with female longevity in the herd (Johnston et al., 2009; Buzanskas et al., 2010; Corbet et al., 2011; Santana et al., 2012; Corbet et al., 2013). In other words, the selection for higher SC and/or higher PNS in young bulls, may lead to female progeny that will be sexually precocious and have a higher probability to stay in the herd. Female fertility traits are of high relevance for beef cattle production in tropical regions, where *Bos indicus* and its crosses are prominent. Female fertility in *Bos indicus* and its crosses causes frequent concern because *Bos indicus* cows tend to be older at puberty and have a prolonged port-partum anoestrus (Abeygunawardena and Dematawewa, 2004). Functional mutations identified for female puberty or post-partum anoestrus would be ideal markers for these traits. In their absence, functional mutations identified for SC and PNS could have an effect associated to female reproductive traits. In the context of genetic correlations (~0.3), we also tested the identified functional mutations in female populations. These cows were from the same populations as those used in the original GWAS (Hawken et al., 2012). As the genetic correlations between SC and AGECL and medium (~0.30) some mutations could be associated with both. Testing of female traits was an additional activity carried (beyond initial project plan). Functional mutations hold the promise of being ideal genetic markers. Previous work has shown that many markers with small effects are involved in reproduction and pointed to top markers that will work across breeds, explaining a sizeable proportion (between 9 and 29%) of the genetic variation (Snelling et al., 2012; Fortes et al., 2013a; Snelling et al., 2013). Both identification and validation of functional mutations associated to SC and PNS were the objective of the proposed project. Identification of functional mutations was via combination of GWAS and genome sequence. The following sections will detail the identification and association results for 13 putative functional mutations.

2 Project objectives

- Identified at least 10 potential functional mutations to be tested for association with scrotal circumference measurements (at 12, 18 and 24 months of age) and percentage of normal sperm.
- Tested the association of the potential functional mutations with male reproductive traits in an independent population of mixed-breed bulls, thereby indicating the success of the approach to identifying ideal markers.
- Genotyped (74k Illumina SNP chip) the 1,000 mixed-breed bulls of the Beef CRC population and performed a genome-wide association study and genomic selection analysis using the new population.
- Integrated the newly genotyped population into the Beef CRC dataset, contributing to improved genomic selection accuracies.
- Assessed the need for, and likely benefit of, additional R&D seeking functional markers related to female reproduction traits.

3 Methodology

3.1 Animals and phenotypic data

Data from 1,021 bulls whose breeds were Brahman (n = 113), Tropical Composite (n = 741) and crossbreeds (n = 167) from five properties born from 2004 to 2009 were used in the current study. These animals were bred by the Beef CRC and the experimental design as well as the general population description of the CRC were reported previously (Burns et al., 2013; Corbet et al., 2013). Importantly, the animals used in this study were not genotyped for any of the previous CRC studies.

The traits utilised in this study were: scrotal circumference at 12 (SC12), at 18 months (SC18) and at 24 months (SC24) months and percentage of normal sperm at 24 months (PNS). Details about the measurements of the traits are available in previous reports (Burns et al., 2013; Corbet et al., 2013).

Analysis of female cattle was beyond the initial objectives proposed for this project. However, we have carried an additional activity where we have used data from 1,089 Tropical Composite and 935 Brahman cows measured for observation of the first corpus luteum (AGECL) and post-partum anoestrus interval (PPAI). Details regarding the female populations and its phenotypes can be found in previous reports (Johnston et al., 2009; Johnston et al., 2010; Hawken et al., 2012).

3.2 Bioinformatic analyses of sequenced genomes

The genomes of 69 bulls (from CSIRO Animal, Food and Health Sciences in St Lucia, Brisbane, QLD, Australia) were used to generate files with information on the mutations present in the target regions, such as SNPs or indels (mutations are also termed variants). Variant Effect Predictor (VEP) from Ensembl website was used to predict the functional consequences of detected mutations. The aim was to find disruptive mutations with a major effect on the traits. We therefore focused on the identification of non-synonymous SNPs and SNPs/indels in codifying regions and splicing sites of the candidate genes.

3.3 Genotyping of selected SNPs

Custom TaqMan assays were developed for the novel selected SNPs according to TaqMan Array Design Tool. The genes targeted with novel custom assays were: *LOC100138021, CENPI, TAF7L, NXF2, CYLC1, UXT, SPACA5* (in chromosome X) *HELB*, *INHBC* and *FAU* (in chromosome 5). For SNPs in *TEX11*, *AR* and *PLAG1* genes, primers and probes were used as described by the original publications of Lyons et al. (2014a) and Karim et al. (2011), respectively.

In addition to the contracted work for this project, we have genotyped female cattle for some of the new SNP markers. For the mutations in all the X chromosome genes and for the *PLAG1* SNP both Brahman (n = 935) and Tropical Composite (n = 1,089) females were genotyped. Tropical Composite females (n= 1,089) were genotyped for SNPs in *FAU*, *HELB* and *INHBC*. As these mutations were only genotyped and tested in Tropical Composite females, further funding and investigation will be necessary to determine their significance for AGECL and PPAI in Brahman cows.

3.4 Genotyping with genome-wide SNP panel

The SNP chip commercialised by Geneseek® was used to genotype 1,021 bulls for 74,000 genetic markers distributed across the genome. This chip uses the Illumina platform and genotyping service was provided by Animal Genetics Laboratory (AGL), at the University of Queensland, Gatton. Genotype calling and quality control were performed using Genome Studio (Illumina, 2015). Manual editing was required for all SNP in the X chromosome because the software is unable to account for lack of heterozygous and this is an all-male population. After quality control, genome-wide genotyping was available for 1,012 bulls. Only 9 bulls had poor call rates and their genotype information had to be discarded.

3.5 Analyses of SNP association to phenotypes

The association of each SNP with SC12, SC18, SC24, PNS, AGECL and PPAI was examined for genotyped animals using a mixed model analysis of variance with ASREML software. The mixed model included fixed effects, random additive polygenic effects of animal (pedigree relationship matrix) and the observed animal genotype for the SNP (coded as 0, 1 or 2 to represent the number of copies of the B allele) and a random residual effect. The same fixed effects were used for each trait. These fixed effects included contemporary group (animals born in the same year and raised together) and breed. Age was fitted as a covariant in the model of SC measurements and PNS. This model was used for both target SNP genotypes and GWAS analysis.

3.6 Genomic selection analyses

The aim of performing genomic selection analysis was to verify the benefit of adding the bulls genotyped in this project to the Beef CRC database. Benefit was measured in terms of improvement in genomic selection accuracy of the studied phenotypes. To this aim, the data analysed spanned three MLA projects: **B.NBP.0604** (1,115 Brahman bulls), **B.NBP.0723** (1,019 Tropical Composite bulls) and **B.NBP.0786** (current project, 1,012 mixed-breed bulls).

Genomic predictions were performed with Bayes R (Erbe et al., 2012). The analysis was performed using phenotypes adjusted for the same fixed effects described in in the GWAS section. Accuracy of genomic selection was estimated using a cross-validation approach. Under this methodology sires were allocated at random to one

of four groups. This approach means that animals in validation will not have any sire siblings used in training the prediction equations. The cross-validation was performed by assigning each animal to a group based such that no sires were represented across groups. The predictions were then generated using 3 out of 4 groups to train the equations then validating on the remaining group. In this manner each scenario explored had four results. Reported results represent the averages of the cross-validation analyses.

There were four training scenarios explored, with cross-validation based on sire family splits for each. The four training scenarios were: Scenario 1 (ALL) All animals included in training; Scenario 2 (MLA-valid) the new animals genotyped by this project were only used for validation and not used at all in training; Scenario 3 (BB) Only Brahman bulls were used in training; Scenario 4 (TC) Only tropical composite bulls were used in training. Brahman bulls in scenario 3 included Brahmans genotyped previously and Brahmans newly genotyped by this project. Likewise, scenario 4 included Tropical Composite bulls from this and previous projects.

For validation, data was also divided into animal groups to represent each research project. The first group consisted of the animals genotyped in **B**.**NBP.0604**, which included only Brahman bulls not genotyped in the current project. The second group was formed by bulls genotyped in **B.NBP.0723**, which were all Tropical Composites. The third group were the animals genotyped in **B.NBP.0786**, the current project, which formed the mixed-breed population.

The sensitivity of the results to trait and diversity was examined by first running a model with all the data as potential training data. Then the training data was put into subsets as follows. Breed specific training (training within only BB or TC animals) and lastly the newly genotyped animals were used only for validation of the prediction equations. The training scenarios and validation approaches were designed to answers 3 research questions:

(1) Does accuracy increases by adding more animals into the genomic prediction training sets?
(2) What is the accuracy of prediction across breeds?
(3) Is it possible to validate the predictive potential of the original datasets in a separated population?

Further, a likelihood test was applied to verify if the addition of functional mutations to pedigree based of genomic EBVs had a significant impact, in terms of genetic variance explained.

4 Results

The research project was executed in two phases. Phase one was dedicated to bioinformatics analysis of the sequenced genomes to find the putative functional mutations associated with components of bull fertility – SC and PNS. These mutations were then tested in an independent population of bulls as a proof of concept. Additionally, mutations were tested in females to estimate their effect in genetically correlated traits. Significant association (P-value < 0.05) with SC and PNS were confirmed for 10 mutations in the validation exercise with males. Out of those 10, only 1 mutation was also associated with female reproductive traits. After successful validation of identified mutations, phase two of the project was initiated. Phase two delivered genome-wide genotyping and analysis for this new mixed-breed population of bulls. Complete genome-wide analysis fully integrated the mixed-breed

population into the Beef CRC legacy database. This additional resource increased the number of phenotyped and genotyped animals and with that improved genomic selection accuracies (1 to 4% higher). Therefore, the validation of top markers and the genome-wide analysis we were able to carry out on these bulls provides valuable information for the future commercial roll-out of the genomic prediction technology to the industry, specifically for reproductive traits in tropical beef breeds.

The results section is divided in 3 parts that further detail the three major outcomes of the project: 1) results related to functional mutations, 2) GWAS results for the mixed-breed bull population genotyped by this project, and 3) genomic selection results, using data from this and other 2 previous projects - **B.NBP.0604** (1,115 Brahman bulls), **B.NBP.0723** (1,019 Tropical Composite bulls) and **B.NBP.0786** (current project, 1,012 mixed-breed bulls).

4.1 Functional mutations: identification and associations

Previously identified “hotspot” regions in the genome, or QTL, for SC and PNS were located in chromosomes 5 and X. These regions were mined for non-synonymous and/or potentially deleterious SNPs that could be putative functional mutations. Non-synonymous SNP were discovered in 10 candidate genes, 3 located in chromosome 5 and 7 in chromosome X (Table 2). Candidate genes were selected based on their location within the QTL regions and because of known biological effects that could influence reproductive physiology (literature annotation of gene function).

Table 2. Description of candidate genes and tested SNP (UMD3.1 positions).

Gene	Gene position (chromosome - bp)	SNP location (chromosome - bp)	NCBI SNP number	SNP Variation	Position in CDS	Position in protein	Amino acid substitution
FAU	5:47,736,728..47,737,193	47737052	Not available	C/T	299	100	T/M
HELB	5:47,713,520..47,751,430	47748370	rs432042680	G/A	536	179	T/M
INHBC	5:56,340,056..56,350,443	56340186	rs134364749	G/A	953	318	T/I
LOC100138021	X:49,737,023..49,737,868	49,737,296	rs461402021	G/C	573	191	I/M
CENPI	X:54,969,324..55,038,297	54,971,267	rs134782295	G/A	143	48	S/N
*TAF7L**	X:55,127,019..55,144,665	55,133,975	rs445729496	C/T	829/535	277/179	A/T
NXF2	X:55,592,336..55,604,610	55,602,546	ss1026566625	T/C	661	221	N/D
CYLC1	X:69,903,617..69,933,552	69,914,225	rs477320469	T/A	818	273	Y/F
UXT	X:91,468,065..91,474,474	91,472,521	rs132821996	C/T	344	115	S/N
SPACA5	X:92,799,368..92,801,705	92,801,539	rs211186307	C/T	109	37	G/S

*TAF7L has splice variants, which explain the 2 positions provided for the amino acid substitution.

The newly discovered non-synonymous SNP from Table 2 plus the previously identified SNP in *TEX11*, *PLAG1* and *AR* were tested in association analysis. The substitution allelic effect for the named allele of each SNP and its standard error, the p-values and the percentage of the additive genetic variance explained were estimated for each studied trait: production of normal sperm (PNS), scrotal circumference at 12 (SC12), at 18 months (SC18) and at 24 months (SC24) (Table 3).

Table 3. Association results for male reproductive traits: 1,021 bulls in the mixed-breed population genotyped*.

Trait	*Gene (SNP location)*	*P PNS*	*P SC24*	*P SC18*	*P SC12*
PNS	*FAU*	0.9089	0.5772	0.2815	0.605
PNS	*HELB*	0.1221	**$1.94x10^{-11}$**	**$5.23x10^{-7}$**	0.0699
PNS	*INHBC*	0.0758	0.4217	0.786	0.9307
PNS	*PLAG1*	0.3075	0.1205	0.0408	0.0308
PNS	*LOC100138021*	0.0001	**$1.68x10^{-05}$**	**$2.33x10^{-08}$**	**$1.09x10^{-11}$**
PNS	*CENPI*	0.0007	0.0094	**$7.66x10^{-06}$**	**$1.99x10^{-06}$**
PNS	*TAF7L*	0.0003	**$4.54x10^{-06}$**	**$5.88x10^{-10}$**	**$2.36x10^{-13}$**
PNS	*NXF2*	0.0192	0.0104	0.0029	**$7.93x10^{-05}$**
PNS	*CYLC1*	0.2842	**$3.07x10^{-08}$**	**$3.78x10^{-07}$**	**$2.14x10^{-07}$**
PNS	*TEX11*	0.2131	**$3.59x10^{-19}$**	**$1.65x10^{-16}$**	**$3.87x10^{-15}$**
PNS	*AR*	0.0791	**$1.01x10^{-13}$**	**$2.33x10^{-10}$**	**$1.36x10^{-09}$**
PNS	*UXT*	0.0807	$1.29x10^{-05}$	0.0011	0.0172
PNS	*SPACA5*	0.3796	0.112	0.0067	0.0928

*Significance (P) for each SNP on percentage of normal sperm (PNS), scrotal circumference at 12 months (SC12), at 18 months (SC18) and at 24 months of age (SC24). Highlighted in bold are SNP that had a *P*-value lower than 10^{-5}, considered the most significant results.

Considering genetic correlations between male and female fertility traits observed for the Beef CRC populations in previous studies (Johnston et al., 2013b; Wolcott et al., 2013), we also genotyped female cattle for the discovered functional mutations. Genotyping of female cattle was beyond the objectives proposed for this project. As an additional activity we have genotyped 1,089 Tropical Composite females for *FAU*, *HELB* and *INHBC*. As these mutations were only genotyped and tested in Tropical Composite females, further investigation is necessary to determine their significance for AGECL and PPAI in Brahman cows. For the mutations in all the X chromosome genes and for the *PLAG1* SNP both Brahman (n = 935) and Tropical Composite (n = 1,089) females were genotyped. For AGECL, *PLAG1* continues to be the most significant association found and validated across breeds. The mutation in *HELB* had some association with AGECL (*P* = 0.0029) and a highly significant association with PPAI (*P* = $2.15x10^{-8}$). Except for *PLAG1* and *HELB*, no other mutations were associated with the studied female traits (Table 4).

Table 4. Association results for female reproductive traits, using all cows genotyped for each SNP*.

	AGECL		PPAI	
Gene (SNP location)	***P* BB**	***P* TC**	***P* BB**	***P* TC**
FAU	NA	0.7673	NA	0.7677
HELB	NA	0.0029	NA	**$2.15x10^{-8}$**
INHBC	NA	0.8470	NA	0.8530
PLAG1	**$1.92x10^{-10}$**	**$7.09x10^{-6}$**	$1.86x10^{-2}$	$4.15x10^{-3}$
LOC100138021	0.382	0.588	0.138	0.579
CENPI	0.462	0.556	0.574	0.281
TAF7L	0.272	0.498	0.224	0.467
NXF2	0.373	0.86	0.907	0.331
CYLC1	0.861	0.29	0.616	0.369
TEX11	0.932	0.775	0.468	0.278
AR	0.022	0.747	0.665	0.485
UXT	0.062	0.918	0.316	0.516
SPACA5	0.196	0.874	0.19	0.823

*Significance (P) for each SNP on age at observation of the first corpus luteum (AGECL) and post-partum anoestrus interval (PPAI). Females genotyped were Brahman (BB) and Tropical Composite (TC) cows from the Beef CRC populations used in previous GWAS (Hawken et al., 2012). For chromosome 5 mutations, only TC cows were genotyped and so results for BB are not available (NA). Highlighted in bold are SNP that had a *P*-value lower than 10^{-5} considered the most significant results.

4.2 GWAS results

After quality control 74,251 SNP across the genome were available for association analyses in the mixed breed population. Analyses were run first with all the animals (ALL), irrespective of breed and then separately for each breed: Brahman (BB), Tropical Composites (TC) and crossbreeds (XB); as summarised in Table 5.

Genome-wide SNP associations were calculated with and without functional mutations in the model to provide evidence that these mutations could explain the observed QTL for SC measurements in chromosomes 5 and X. For chromosome X, the functional mutation fitted in the model was the *TEX11* SNP (Figure 1). The functional mutation in HELB was fitted for chromosome 5 (Figure 2). The lowering of P-values in chromosome X is evident when *TEX11* is in the model. The same degree of overall P-value reduction occurs when *HELB* is in the model, although this is smaller than what was observed for *TEX11*. These results confirm the previously identified QTL in chromosomes 5 and X and provide further evidence for the relative significance of the functional mutations in *TEX11* and *HELB*. As these mutations were not associated with PNS (Table 3), the same plot was not produced for PNS (i.e. no difference is expected for traits that showed no association). Figure 3 presents the overall results for the PNS in a classic GWAS Manhattan, with no adjustment for *TEX11* or *HELB*. Chromosomes X and 21 showed SNP associations for PNS in the mixed breed population of bulls.

Table 5. Summary of genome-wide association studies: number of SNP associated to percentage of normal sperm and scrotal circumference at 8 *P*-value thresholds.

Breed	Trait	***P*-values**							
		$5x10^{-2}$	$1x10^{-2}$	$1x10^{-3}$	$1x10^{-4}$	$1x10^{-5}$	$1x10^{-6}$	$1x10^{-7}$	$1x10^{-8}$
ALL	*PNS24*	8,902	2,128	322	64	4	1	0	0
	SC12	11,051	4,045	2,001	1,490	1,137	908	702	543
	SC18	20,003	9,465	4,139	2,435	1,758	1,400	1,169	1,004
	SC24	10,804	3,945	1,836	1,282	1,038	859	737	643
BB	*PNS24*	12,328	3,654	638	110	14	2	0	0
	SC12	2,050	132	3	0	0	0	0	0
	SC18	17,080	6,791	1,810	461	110	31	12	3
	SC24	7,072	1,362	114	5	0	0	0	0
TC	*PNS24*	7,221	2,037	694	353	160	74	4	2
	SC12	11,671	4,405	2,226	1,679	1,398	1,186	1,004	786
	SC18	19,304	8,984	3,932	2,430	1,785	1,465	1,255	1,087
	SC24	10,827	4,055	1,978	1,390	1,093	919	767	633
XB	*PNS24*	13,361	4,176	789	159	36	3	0	0
	SC12	9,292	2,135	189	17	0	0	0	0
	SC18	17,779	7,146	1,918	519	87	19	5	0
	SC24	8,259	1,941	389	32	1	0	0	0

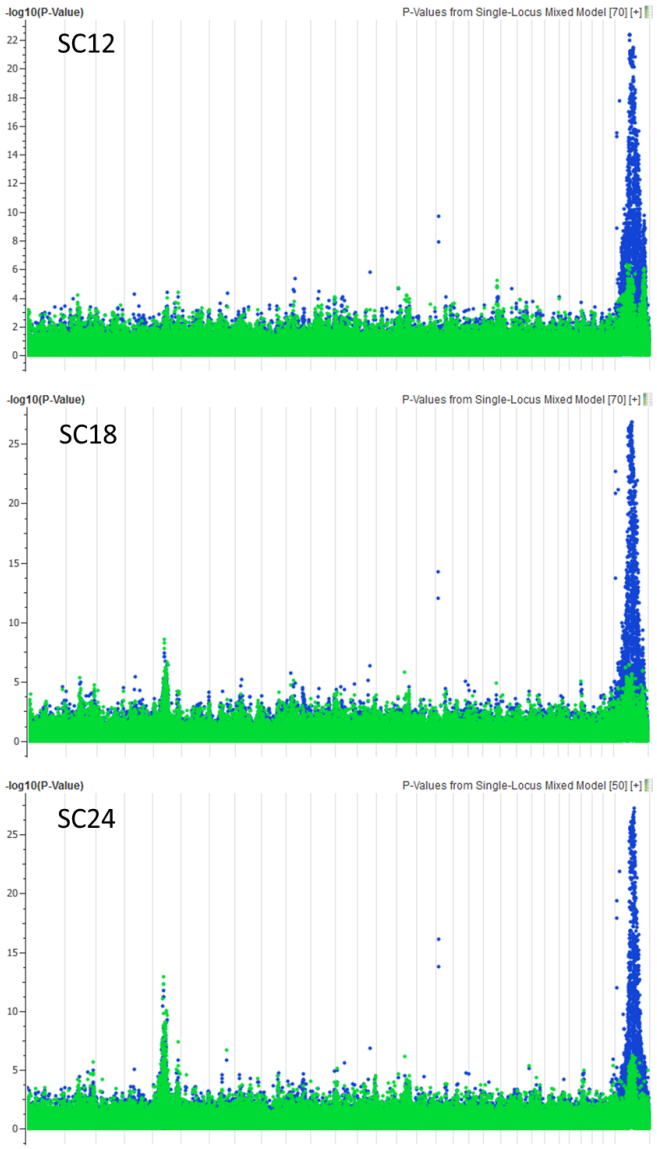

Figure 1. Manhattan plots representing genome-wide association results for scrotal circumference measurements at 12, 18 and 24 months of age (SC12, SC18 and SC24). In the *y*-axis are –log10 *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 reference genome). Data points in blue represent the SNP associations from the initial model, without the *TEX11* mutation. Data points in green represent the SNP associations from the model where the *TEX11* mutation was fit as a fixed effect.

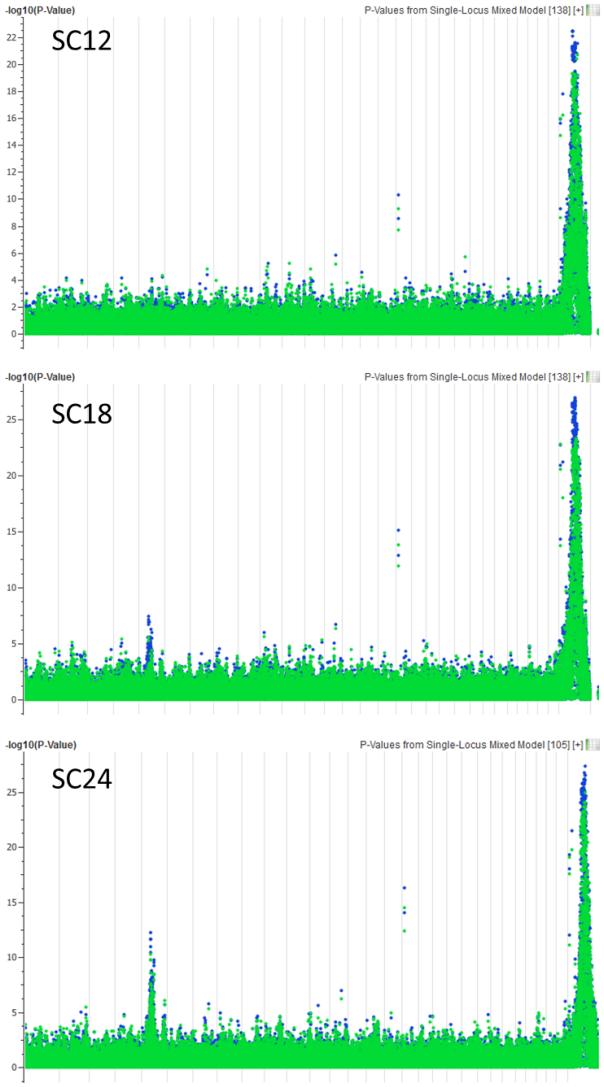

Figure 2. Manhattan plots representing genome-wide association results for scrotal circumference measurements at 12, 18 and 24 months of age (SC12, SC18 and SC24). In the *y*-axis are –log10 *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 reference genome). Data points in blue represent the SNP associations from the initial model, without the *HELB* mutation. Data points in green represent the SNP associations from the model where the *HELB* mutation was fit as a fixed effect.

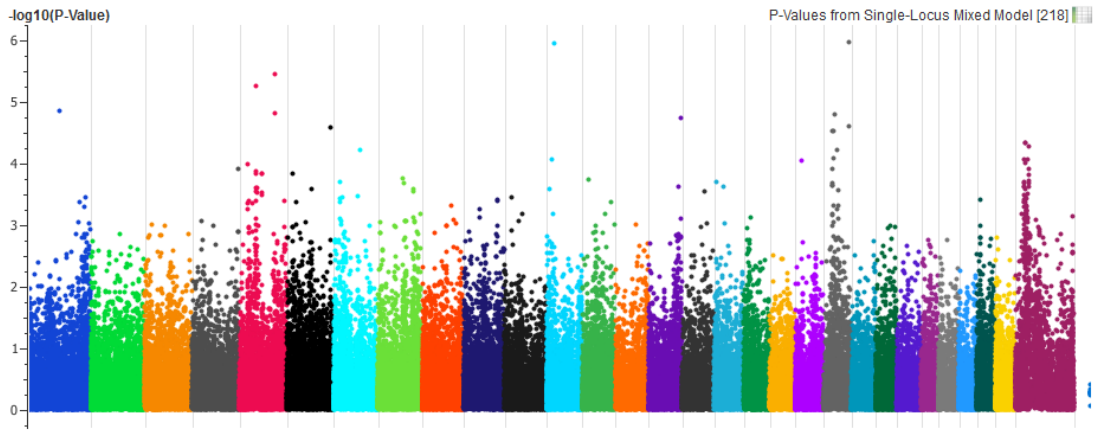

Figure 3. Manhattan plot representing genome-wide association results for percentage of normal sperm measured at 24 months of age (PNS24). In the *y*-axis are –log10 *P*-values and in the *x*-axis are chromosomal positions (UMD3.1 reference genome). Data points in represent the SNP associations from the GWAS model.

4.3 Genomic selection results

Genomic selection analysis results were different for SC measurements and PNS. The use of a multi-breed training population resulted in correlations between estimated breeding values and phenotypes that were best than single breed training for SC measurements, or at least not worst (Table 6). The same multi-breed training strategy did not produce improved accuracies for PNS (Table 6). Correlations between estimated breeding values and phenotypes were provided as a measurement of genomic selection accuracy.

Table 6. Correlations (r) between estimated breeding values and adjusted phenotypes (average from cross-validation sire groups). Training data was divided in four scenarios and validation data was divided in 3 groups.

	Validation Group		
Training Group	B.NBP.0604 (BB)	B.NBP.0723 (TC)	B.NBP.0786 (mixed-breeds)
SC18			
ALL	0.36	0.49	0.44
MLA-valid	0.35	0.45	0.41
BB	0.35	0.28	0.22
TC	0.35	0.45	0.41
SC24			
ALL	0.38	0.49	0.46
MLA-valid	0.38	0.45	0.44
BB	0.39	0.29	0.26
TC	0.21	0.40	0.37
PNS24*			
ALL	0.03	0.20	0.05
MLA-valid	0.08	0.19	0.08
BB	0.11	0.12	0.04
TC	-0.10	0.22	0.07

Training data: 1) All animals included in training (ALL); 2) Animals genotyped by this project were only used for validation and not used in training (MLA-valid); 3) Only Brahman bulls were used in training (BB); and 4) Only Tropical Composite bulls were used in training (TC).
Validation data: 1) B.NBP.0604 consisted of 1,115 Brahman animals; 2) B.NBP.0723 consisted of 1,019 Tropical Composite; and 3) B.NBP.0786 consisted of 113 Brahman, 741 Tropical Composites and 167 crossbreeds.
*For the purpouse of this project we have estimated a gEBV for PNS24, although no EBV for PNS24 is currently commercially available.

Table 7. Percentage of variance explained by 11 functional mutations (FM) independently or as part of an animal model.

Model*	**SC12**	**SC18**	**SC24**	**PNS24**
FM only	5.46	5.32	5.51	0.07
FM + A	5.17	4.16	4.62	0.22
FM + GRM	4.39	3.51	4.37	0.19
GRM-X	1.74	0.39	1.17	0.00

*FM only: model included only functional mutations (11 markers) and fixed effects; FM + A: model included a pedigree matrix, functional mutations and fixed effects; FM + GRM: model included genomic relationship matrix from autosomes (no chromosome X), functional mutations and fixed effects; FM + GRM-X model included genomic relationship matrix from autosomes and chromosome X, functional mutations and fixed effects. In most models for prediction of scrotal circumference, the addition of the functional mutations had significant impact ($P < 0.001$, see Table 8). The genetic variance explained by these mutations was higher for models that did not include the X chromosome, which are commonly used in current EBV predictions.

Table 8. Significance (likelihood ratio test *P*-value) for the addition of the functional mutations (FM) to pedigree and genomic models.

Model*	SC12	SC18	SC24	PNS24
FM + A	<0.001	<0.001	<0.001	0.3222
FM + GRM	<0.001	<0.001	<0.001	0.4166
FM + A + GRM	<0.001	<0.001	<0.001	0.3125
A + GRM	<0.001	<0.001	<0.001	0.5967
		GRM-X		
FM + GRM-X	0.0190	0.4543	0.0991	1.0000
FM + A + GRM-X	0.0190	0.4543	0.0644	0.7290
A + GRM-X	<0.001	<0.001	<0.001	0.1492

*FM + A: in this model we tested the effect of including the FM matrix in the traditional pedigree model; FM + GRM: in this model we tested the effect of including the FM matrix in the genomic model where the genomic matrix had no chromosome X data; FM + GRM-X: in this model we tested the effect of including the FM matrix in the genomic model where the genomic matrix had chromosome X data; A + GRM: in this model we tested the effect of including the pedigree matrix in the genomic model where the genomic matrix had no chromosome X data; A + GRM-X: in this model we tested the effect of including the pedigree matrix in the genomic model where the genomic matrix had chromosome X data.

5 Discussion

5.1 Functional mutations

Functional mutations were discovered in 10 genes, the majority of which (7) were located in the X chromosome. The associations shown for the SNP in *TEX11* and other functional mutations indicate that the newly genotyped bulls were adequate as a validation resource. Further, these associations serve to prove the concept that discovery of functional mutations can be guided by GWAS and sequenced genomes in the combined approach used herein. For SC, in the X chromosome the gene *TEX11* was the most promising candidate gene out of this exercise, confirming previous results (Lyons et al., 2014b). For PNS, *LOC100138021* and *CENPI* had the highest associations, yet these associations do not fully explain the QTL previously

identified for PNS. For PNS, additional discovery of functional mutations in the X chromosome would be an avenue for future research. The X chromosome in mammals is densely populated with miRNA that are highly expressed in spermatocytes and spermatids (Meunier et al., 2013). Exploring the role of miRNA in relation to bull reproductive traits is another potential area for future work.

Other functional mutations discovered were non-synonimous SNP within the genes *FAU*, *HELB* and *INHBC*. These genes mapped to the QTL location on chromosome 5 and the mutation in *HELB* showed significant associations with SC18 and SC24, but not SC12. In context with genome-wide markers, the SNP in *HELB* did not produce the same reduction in *P*-values for the QTL region than what could be expected from a causative mutation, as observed for *TEX11* herein and for *PLAG1* in previous work (Fortes et al., 2013a). Therefore, additional work exploring the QTL in chromosome 5 is warranted.

Except for *PLAG1, AR* and *HELB*, no other mutations were associated with the studied female traits: AGECL and PPAI. The *G* allele of *PLAG1* decreased AGECL and increased SC12. Although *PLAG1*'s impact in males is more evident if age at puberty is calculated from multiple SC measurements (Fortes et al., 2013a), our results confirm that *PLAG1* can be used for selection for reproductive traits in males and females, with favourable correlations. Because there was no association between SNP and female traits, the mutations in *TEX11, LOC100138021, CENPI, TAF7L, NXF2,* and *CYLC1* could be used in bull selection for bigger SC, without detrimentally affecting female reproductive traits. The effect of the SNP in *HELB* needs to be confirmed in Brahman females in future work. Also, the impact of this *HELB* SNP in the QTL on 5 for PPAI needs to be tested in the context of genome-wide markers (similar to the analysis reported here for male traits).

5.2 Genome-wide association study

The GWAS performed in this mixed-breed cattle population confirmed the QTL in the X chromosome identified in previous GWAS performed in Brahman and Tropical Composites (Fortes et al., 2012a; Fortes et al., 2013b). It also confirmed the QTL in chromosome 5, which was previously identified only for Tropical Composites. The majority of the animals genotyped herein were Tropical Composites and so it is highly possible that chromosome 5 continues to be more relevant for this breed. The finding that the chromosome 5 QTL was present for SC18 and SC24, but not SC12 is in agreement with the associations found for the SNP in *HELB*. The SNP in *HELB* is probably in high linkage disequilibrium (LD) with the causative mutation underpinning this QTL.

5.3 Genomic selection across Brahman and Tropical Composites

The data used in the genomic selection analyses spanned three MLA projects: **B.NBP.0604** (1,115 Brahman bulls), **B.NBP.0723** (1,019 Tropical Composite bulls) and **B.NBP.0786** (current project, 1,012 mixed-breed bulls). Together, these three projects have genotyped the vast majority of the Beef CRC bull population referred to as *"male indicators of female reproductive traits"*. This population was intensively phenotyped and represents a very important resource for genetics and genomics of tropical beef cattle.

Genomic selection was performed using Bayes R because this method was shown to have a slightly higher correlation between in multi-breed beef data than GBLUP (Bolormaa et al., 2011). Bayes R assumes that SNP effects come from a series of normally distributions from those explaining 0% of the variance through to a

distribution explaining 1% of the variance. The analysis was run for 50,000 iterations with the first 10,000 discarded as burn in. The variances of individual SNPs within each group were fixed to increasing proportions of the genetic total variance (0, 0.01, 0.1 and 1% of the total genetic variance) (Erbe et al., 2012).

The training scenarios and validation approaches used in genomic selection were designed to answers 3 research questions: 1) Does accuracy increases by adding more animals into the genomic prediction training sets?; 2) What is the accuracy of prediction across breeds?; and 3) Is it possible to validate the predictive potential of the original datasets in a separated population? This discussion will cover the answers to these questions given the presented results.

The accuracy of genomic selection can be improved by adding genotyped and phenotyped animals to the dataset. This was achieved in the ALL scenario for SC, although the same result was not verified for PNS. The conclusion is that not always adding more animals to the training population will be beneficial. Some traits such as PNS seem to be “breed” sensitive. The accuracy of predictions across breeds and using multi-breed training sets was never problematic for SC measurements, but provided lower accuracies for PNS. The accuracies of predictions across breeds was lower than within breed. For example, SC18 Brahman predictions for a Brahman population had an accuracy of 0.35, for Tropical Composites it was 0.28 and for the mixed-breed population it was 0.22. This is expected and similar results were found by other groups, reinforcing the importance of having all breeds represented in the reference population for genomic selection. Nonetheless, it was possible to use the “new” population (mixed-breeds) as a validation resource for genomic selection and for the association analyses, in the validation of functional mutations.

Further, we used genomic selection predictions to evaluate the merit in adding functional mutations to pedigree based EBVs and genomic predictions (gEBVs). For SC measurements, functional mutations added to both the pedigree model and the genomic model were significant. Functional mutations explained additional genetic variance in SC predictions, which contributes to model accuracy. To expand this positive result to PNS and other traits of interest (such as female fertility traits) additional discovery of associated functional mutations is needed.

6 Conclusion

We have demonstrated that combining GWAS and sequenced genomes is a valid approach to identify functional mutations that were validated in an independent dataset. The highly significant mutations mapped to *TEX11*, *HELB, LOC100138021, CENPI, TAF7L* and *PLAG1* should be included in future marker panels aimed at improved selection for reproductive traits, especially SC, in tropical Beef cattle. The mutation in *HELB* would also benefit female reproductive traits as it was directly associated with PPAI and AGECL.

The additional animals genotyped in this project will continue to serve the commercial roll-out of genomic predictions via BREEDPLAN as this dataset was incorporated in the Beef CRC legacy database. The addition of newly genotyped animals serves as validation for genomic predictions and/or to increase the training dataset, resulting in increased accuracies.

7 Recommendations and future directions

The knowledge gathered in this and previous research projects points to functional mutations that are tools in genomic selection, especially discovered for tropically adapted breeds.

To further this area of research future work could aim at:

- Discovery of additional functional mutations both in male and female cattle. The functional mutations herein did not explain all confirmed QTL and so there is need for additional discovery exercises.

- The fact that the X chromosome harbours the most promising results for bull reproductive traits aligned with the knowledge that miRNA genes on the X have higher expression levels than all other miRNA categories in male germ cells (Meunier et al., 2013), suggest that miRNA should be studied and tested for their association with PNS and SC.

- Discovery of functional mutations needs to move to applied research. New technology is now available to construct reduced SNP panels that could be more cost effective. This new technology is based on genotype by sequencing with next-generation sequencing. Testing this new technology by genotyping phenotyped animals for the discovered functional mutations will be an important step before commercial implementation of a reduced SNP panel, focussed on selection of tropically adapted cattle.

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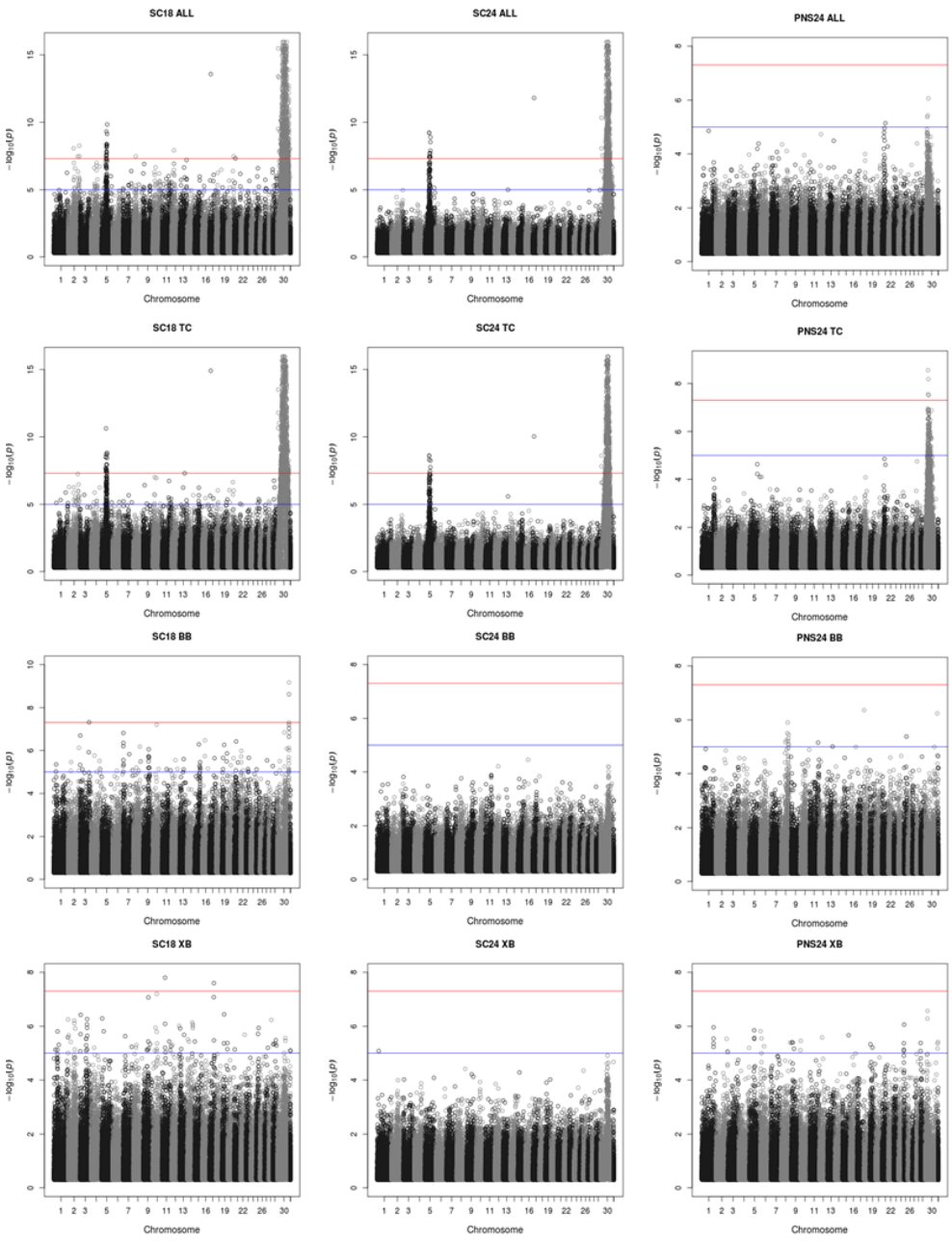
9 Appendices

As an appendix to this report we provide Manhattan plots for SC18, SC24 and PNS24 for GWAS carried separately for each breed of the mixed-breed population (Appendix Figure 1). This GWAS analysis per breed is not part of the main report as we would recommend caution in interpretation of GWAS results for Brahman (n=113) and crossbreeds (n=167), given the low number of animals in this datasets.



Appendix Figure 1. Genome-wide associations carried in all animals (ALL) and then separately for each breed in the mixed breed population of bulls: Brahman (BB), Tropical Composites (TC) and crossbreeds (XB).

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