# Gene network analyses of first service conception in Brangus heifers: Use of genome and trait associations, hypothalamic-transcriptome information, and transcription factors[1]

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**ABSTRACT**: Measures of heifer fertility are economically relevant traits for beef production systems and knowledge of candidate genes could be incorporated into future genomic selection strategies. Ten traits related to growth and fertility were measured in 890 Brangus heifers (3/8 Brahman × 5/8 Angus, from 67 sires). These traits were: BW and hip height adjusted to 205 and 365 d of age, postweaning ADG, yearling assessment of carcass traits (i.e., back fat thickness, intramuscular fat, and LM area), as well as heifer pregnancy and first service conception (FSC). These fertility traits were collected from controlled breeding seasons initiated with estrous synchronization and AI targeting heifers to calve by 24 mo of age. The BovineSNP50 BeadChip was used to ascertain 53,692 SNP genotypes for ~802 heifers. Associations of genotypes and phenotypes were performed and SNP effects were estimated for each trait. Minimally associated SNP ($P < 0.05$) and their effects across the 10 traits formed the basis for an association weight matrix and its derived gene network related to FSC (57.3% success and heritability = 0.06 ± 0.05). These analyses yielded 1,555 important SNP, which inferred genes linked by 113,873 correlations within a network. Specifically, 1,386 SNP were nodes and the 5,132 strongest correlations ($|r| \geq 0.90$) were edges. The network was filtered with genes queried from a transcriptome resource created from deep sequencing of RNA (i.e., RNA-Seq) from the hypothalamus of a prepubertal and a postpubertal Brangus heifer. The remaining hypothalamic-influenced network contained 978 genes connected by 2,560 edges or predicted gene interactions. This hypothalamic gene network was enriched with genes involved in axon guidance, which is a pathway known to influence pulsatile release of LHRH. There were 5 transcription factors with 21 or more connections: *ZMAT3*, *STAT6*, *RFX4*, *PLAGL1*, and *NR6A1* for FSC. The SNP that identified these genes were intragenic and were on chromosomes 1, 5, 9, and 11. Chromosome 5 harbored both *STAT6* and *RFX4*. The large number of interactions and genes observed with network analyses of multiple sources of genomic data (i.e., GWAS and RNA-Seq) support the concept of FSC being a polygenic trait.

**Key words:** cattle; first service conception; gene network; genome wide association; heifer


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## INTRODUCTION

There are many well-described factors influencing heifer fertility. *Bos indicus*-influenced heifers typically mature later than *Bos taurus* heifers. However, composites derived from these species are useful in beef production systems in warm climates because of breed complementarity and heterosis (Luna-Nevarez et al., 2010; Riley et al., 2010; Thrift et al., 2010).

Heifer fertility has been reported via numerous descriptors. Luteal phase concentrations of serum progesterone were an indicator of puberty in physiology studies (Day and Anderson, 1998; Shirley et al., 2006), whereas binary traits collected from breeding seasons are typical in genetic evaluations. First service conception (**FSC**) and heifer pregnancy (**HPG**) are examples of such binary traits (Doyle et al., 2000; Cammack et al*.,* 2009; Minick and Wilson, 2010). Reproductive trait data are being collected by breed associations via whole-herd reporting systems for genetic evaluation. The time needed for accrual of ample data challenges this process; thus, genomic information may expedite development of these evaluations (Goddard and Hayes, 2009; Hayes and Goddard, 2010; Van Eenennaam et al., 2011).

Deriving gene networks and pathways from genome wide association studies (**GWAS**) is a growing approach to understand QTL associations and the underlying genes (Schadt, 2009; Butte et al., 2011; Prentice et al., 2011). The biological approach of this system was used to evaluate genotype-to-phenotype associations with age at puberty in heifers from the tropics and revealed candidate genes that would have been missed by GWAS alone (Fortes et al., 2010a,b, 2011). In the current study, we applied this approach with FSC data from Brangus (i.e., 3/8 Brahman × 5/8 Angus) heifers. Genes identified were verified in the transcriptome of the hypothalamus and a resulting network was generated that emphasized transcription factors (**TF**).

## MATERIALS AND METHODS

Animals were handled and managed according to Institutional Animal Care and Use Committee guidelines of New Mexico State University (**NMSU**).

### *Heifers and Traits*

Data were from heifers registered with International Brangus Breeders Association (San Antonio, TX; 3/8 Brahman x 5/8 Angus). As typical of seedstock animals, 890 heifers had various phenotypes. Blood samples were obtained from 855 heifers; 835 heifers had adequate blood sample for DNA extraction and successful genotypes were obtained from an average of 802 heifers. Heifers were raised in 2 locations: Camp Cooley Ranch (Franklin, TX) in east central Texas and the Chihuahuan Desert Rangeland Research Center and Campus Farm of New Mexico State University described by Luna-Nevarez et al. (2010). There were pedigree connections via 5 AI sires that were common to the 2 groups of heifers and numerous other familial relations via historic AI sires registered with International Brangus Breeders Association. The mean percent inbreeding coefficient of these heifers was 8.24 ± 0.001and 67 sires were represented in the data.

Information queried from the databases of Camp Cooley Ranch and NMSU for each heifer included date and year of birth (2005 to 2007); calving season (spring or autumn); pedigree; conception method (AI, embryo transfer, or natural service); BW measured at birth, weaning, and yearling; and yearling ultrasound assessment of LM area, percent fat within LM, and fat thickness over the 12th rib (i.e., backfat). Contemporary group and the date each of these phenotypes were recorded were also extracted from the database. Contemporary groups were constructed with information such as year, calving season, and birth location. Nongenetic effects of age at measurement were taken into account by adjusting BW (i.e., 205 and 365 d of age) and ultrasound measures (i.e., 365 d of age), using formulas from the Brangus Herd Improvement Records (2010). Adjusted BW values were used to calculate postweaning ADG. Specifically, growth traits were measured when heifers were approximately 205 d of age (weaning) and 365 d of age (yearling), and will be defined herein as BW at weaning, BW at yearling, weaning hip height, yearling hip height, postweaning ADG (kg/d), and yearling ultrasound assessment of LM area, backfat thickness, and percent of intramuscular fat in LM.

Fertility trait data were collected from controlled breeding seasons where a heifer had to become pregnant by 15 mo of age as to calve by 24 mo of age. Breeding seasons were ~70 d in length. Breeding seasons began with estrous synchronization and AI based on estrous detection for a minimum of 1 AI service, and then exposure to natural service mating. The spring AI date was typically April 15, analogous to the date used in the NMSU breeding program. Fall breeding season began on the first Monday in November (i.e., average date of Nov. 6). The database included pregnancy via AI or natural service. For the Camp Cooley heifers, these data were determined by pregnancy assessment via ultrasound, calving records, and the nonreturn to estrus from additional AI as some heifers were given 4 AI opportunities. Sire, via AI or natural service, was determined by DNA parentage verification for the calves from the NMSU heifers. Two binary fertility traits were coded from these data, FSC and HPG. All of these data were used to code a success or failure for FSC. Specifically, only heifers that conceived from their first AI service were coded as a success for FSC. If

a heifer conceived from any of these matings by the end of her first breeding season, she was given a success for HPG. Thus, not all heifers that were coded a success for HPG were coded a success for FSC. Also, A heifer must have become pregnant in the same season she was born (spring or autumn) to be coded a success for HPG.

### *DNA and Genotypes*

A single blood sample from each heifer was collected with vacuumized tubes coated with EDTA and shipped to New Mexico State University. Tubes were centrifuged 1875 × *g* for 30 min at 4°C and then white blood cell supernatant (i.e., buffy coat) recovered using procedures described by Beauchemin et al. (2006) and Thomas et al. (2007). The Flexigene kit and procedure (#51204; Qiagen, Valencia, CA) was used to extract DNA from the samples. Genotyping was performed by Advanced Genomics Technology Center (Fairfax, VA). Genotyping used 100 ng of DNA in 100 μL of nuclease-free water per sample and BovineSNP50 (i.e., Infinium BeadChip; Illumina, Inc., San Diego, CA; Matukumalli et al., 2009). Genotype call rates averaged 98.1 ± 0.001% for 53,692 SNP. Genotypes were in the Illumina A/B allele format and were used to compute a value at each locus coded as 0, 1, or 2, representing the number of B alleles. Positions for SNP on the assay were annotated based on the *Bos taurus* genome assembly (Btau_4.0; Liu et al., 2009).

### *Genome Wide Association Study*

Genotype-to-phenotype association analyses were performed, calculating SNP significance and effects for each trait. This initial single-trait-single-SNP GWAS used an animal model to estimate SNP effects. The model was $\mathbf{y} = \mathbf{X\beta} + \mathbf{Z}_a\mathbf{u}_a + \mathbf{e}$, with $\text{var}(\mathbf{u}_a) = \mathbf{A}\sigma_a^2$, $\text{var}(\mathbf{e}) = \mathbf{I}\sigma_e^2$, and $\text{cov}(\mathbf{u},\mathbf{e}) = \mathbf{0}$; $\mathbf{y}$ was a vector of observations, $\boldsymbol{\beta}$ was a vector of fixed effects related to observations by incidence matrix $\mathbf{X}$, $\mathbf{u}_a$ was a vector of random additive genetic effects related to observations by incidence matrix $\mathbf{Z}_a$, and $\mathbf{e}$ was a vector of random residual effects; $\mathbf{A}$ was the numerator relationship matrix between animals computed from pedigree information and $\mathbf{I}$ was an identity matrix; $\sigma_a^2$ represented additive genetic variance and $\sigma_e^2$ represented residual variance. Fixed effects included in the model were dam conception type (natural service and AI, or recipient cow for embryo transfer), age of dam category (years for natural dams with a separate category for recipient cows), and contemporary group. Covariates were inbreeding coefficient, age of heifer (days), and genotype (0, 1, or 2 copies of allele "B") in analysis repeated for each of the 34,894 SNP having minor allele frequency >0.05.

The GWAS analyses used procedures of Snelling et al. (2010) and also outputted means, SE, and narrow sense heritability of each trait. The MTDFREML software controlled by a Perl script was also used to estimate the allele "B" substitution effect and SE for each SNP. Significance of SNP associations was assessed by a 2-tailed *t-test* performed with the TDIST function of Excel software (Microsoft Office, Redmond, WA), using the absolute value of the allele effect divided by SE (degrees of freedom, relative to the number of animals genotyped). These results were also –log transformed for visualization in Manhattan plots with Golden Helix software (SVS, Suite 7; Millennium Science Pty Ltd, Surrey Hills, VIC, Australia).

### *Hypothalamic Transcriptome*

The hypothalamus is a regulatory tissue of the reproductive endocrine axis and gene expression data are useful to infer tissue specificity in network analyses (Bliss et al., 2010; Ojeda et al., 2010a,b). In brief, 2 Brangus heifers of close familial relationship (inbreeding coefficient of 35.6%) were used to generate deep-transcriptome (i.e., gene expression) data from the hypothalamus. These 2 heifers were related by pedigree to the heifers evaluated for GWAS and were chosen for transcriptome analyses as to create a resource to verify gene expression in individuals of similar genetic background at reproductive states relevant to this study (i.e., pre and postpubertal).

Heifers were slaughtered in the NMSU Large Animal Abattoir and hypothalamic tissue (1 $cm^3$) was immediately collected and frozen in liquid nitrogen. This tissue included the preoptic and arcuate nuclei regions of the hypothalamus, as used previously by researchers herein for study of growth, reproduction, and appetite physiology (Narro et al., 2003; Thomas et al., 2009). Pubertal status was determined with serum concentrations of progesterone, using procedures of Schneider and Hallford (1996), and Shirley et al. (2006). The hypothalamus was harvested from the pubertal heifer during the luteal phase of the estrous cycle after she experienced 3 estrous cycles. Table 1A describes the physiological characteristics of the heifers.

Tri-Reagent was used to isolate RNA from the entire hypothalamus (i.e., both pre-optic and arcuate nuclei regions), using procedures of Canovas et al. (2010). The RNA were used to construct cDNA libraries, which were sequenced (**RNA-Seq**) using Genome Analyzer II (Illumina, Inc.). Library construction and RNA-Seq were conducted by National Center for Genome Resources (Santa Fe, NM), using procedures of Mudge et al. (2008). Each library was sequenced 6 times for a deep sequence with 1 lane of the chip serving as a control. Procedures yielded transcript sequences 36 bp in

**Table 1.** Physiological and hypothalamic-transcriptome characteristics of a prepubertal and a postpubertal Brangus heifer used for deep RNA-Sequence analyses

| Item | Prepubertal | Postpubertal |
|---|---|---|
| Physiological characteristics at tissue harvest | | |
| Age, d | 325 | 523 |
| BW, kg | 335 | 417 |
| BCS, scale of 1 to 9 | 5.0 | 6.0 |
| Serum progesterone, ng/mL | 0.5 | 5.0 |
| Age at puberty, d | | 436 |
| Ovarian structures | Follicles | Follicles + corpus luteum on left ovary |
| Hypothalamic-transcriptome characteristics | | |
| Total reads, 36mers | 34,264,260 | 37,873,294 |
| Genomic reads aligned, 36mers | 22,280,603 | 26,234,475 |
| Number of annotations | 20,473 | 22,995 |

length (i.e., 36mers), which were assembled to the *Bos taurus* genome (Liu et al., 2009; Ver. 4.0) for transcript interpretation with Alpheus (Miller et al., 2008). This software estimated the frequencies of each aligned read (i.e., numeric data of gene expression in units of reads/million) and annotations of these loci were downloaded within this software by interfacing with the Batch Entrez query tools of National Center for Bioinformatics Information (http://www.ncbi.nlm.nih.gov). For this study, Alpheus was parameterized as un-normalized as the objective of deep sequencing the RNA samples was to obtain information as to the presence or absence of genes expressed in the hypothalamus of either a prepubertal and postpubertal heifer (i.e., comprehensive list of genes expressed in hypothalamus of both heifers), and not a comparison of the expression among the 2 heifers. Table 1B describes the hypothalamic-transcriptome characteristics of the heifers.

### *Association Weight Matrix, Gene Networks, and Transcription Factors.*

First service conception was used in this study as an indicator trait for heifer puberty (Snelling et al., 2012). In brief, an associated weight matrix of 10 growth and fertility traits was used to identify SNP and then various algorithms were used to output genes from important chromosomal loci. These results were then compared with gene information derived from transcriptome analyses of the hypothalamus. Results from these 2 omics analyses were then evaluated for presence of TF. Specifically, construction of an associated weight matrix (**AWM**) started with selection of SNP from GWAS to represent genes in a network. In brief, SNP selection criterion followed the procedures described by Fortes et al. (2010a, 2011) and an associated weight matrix was constructed with 10 traits. A SNP had to be significantly associated ($P < 0.05$) with FSC or a minimum of 2 other traits to be included in the matrix. Column-wise Pearson correlations between FSC and the other 9 traits were calculated using these SNP effect values. The results of these SNP-based correlations were compared with the genetic correlations estimated via WOMBAT software (Meyer, 2007).

Row-wise AWM revealed correlations between SNP effects to predict gene interactions. We studied the predicted gene interactions, using hierarchical clustering, weighted gene network, and pathway analyses to identify genetic drivers of heifer fertility traits. Specifically, the succession of analyses described by Fortes et al. (2010a, 2011) was followed, which used these software programs and algorithms: PermutMatrix (Caraux and Pinloche, 2005), partial correlation and information theory (**PCIT**, Reverter and Chan, 2008), and Cytoscape (Shannon et al., 2003). Network connectivity and centrality analyses identified clusters of highly connected genes and hubs (i.e., 2 SD as a nominal threshold, $P < 0.01$; Tyler et al., 2009). Clusters of highly interconnected genes were identified with MCODE software (Bader and Hogue, 2003). The list of genes in this network was then compared with the list from transcriptome analyses. The network was then filtered by excluding the genes that were not observed in the hypothalamic-transcriptome resource, which combined the information from a prepubertal and postpubertal heifer. Over represented gene ontology terms were identified in these genes, using BiNGO software (Maere et al., 2005) and GOrilla software (Eden et al., 2009). Finally, pathway enrichment analysis was performed with DAVID software (Dennis et al., 2003; Huang et al., 2009).

Genomatix (http://www.genomatix.de) and the atlas of Vaquerizas et al. (2009) were used to identify genes that were TF. Also, using procedures of Fortes et al. (2010a), important TF were selected and promoter regions corresponding to their predicted targets were mined for TF bindings sites (**TFBS**) to verify potential interactions between TF and predicted targets. A hypergeometric distribution test was used to evaluate if the likelihood of finding corresponding TFBS were enhanced after filtering the network with genes expressed in the hypothalamus.

## RESULTS

### *Traits and Correlations*

Table 2 provides descriptive statistics and heritability estimates for the 10 traits measured in Brangus heifers. Table 3 lists the genetic correlations, as well as the Pearson's correlations between SNP effects (i.e., data from GWAS). A correlation estimate of these values suggested these metrics were similar ($r = 0.92$, $P < 0.001$).

**Table 2.** Descriptive statistics and heritability (h2) estimates for growth, ADG, and ultrasound measures of carcass traits in Brangus heifers

| Traits | No. | Mean ± SE | $h^2$ ± SE |
|---|---|---|---|
| 205-d BW, kg | 864 | 240.94 ± 1.00 | 0.48 ± 0.12 |
| 365-d BW, kg | 817 | 361.13 ± 1.44 | 0.48 ± 0.11 |
| 205-d height, cm | 477 | 112.00 ± 0.30 | 0.55 ± 0.15 |
| 365-d height, cm | 477 | 124.40 ± 0.20 | 0.52 ± 0.14 |
| ADG, kg/d | 877 | 1.74 ± 0.01 | 0.28 ± 0.08 |
| LM area,[1] $cm^2$ | 874 | 63.10 ± 0.05 | 0.63 ± 0.11 |
| Intramuscular fat,[1] % | 874 | 4.25 ± 0.03 | 0.42 ± 0.10 |
| Back fat,[1] cm | 874 | 0.60 ± 0.01 | 0.30 ± 0.09 |
| First service conception,[2] % | 861 | 57.30 ± 1.00 | 0.06 ± 0.05 |
| Heifer pregnancy,[2] % | 861 | 78.00 ± 1.00 | 0.07 ± 0.06 |

[1]Adjusted to 365 d of age.

[2]Binary traits measured on yearling heifers (i.e., 1 = pregnant; 0 = non-pregnant). Results presented are pregnancy success.

Note that the fertility traits, FSC and HPG, were positive and moderately associated in correlation analyses derived from both animal model and GWAS approaches. Backfat and FSC was the greatest genetic correlation observed among growth and fertility traits.

### *GWAS, AWM-PCIT, and Transcriptome*

The GWAS results from 10 traits and SNP genotypes are shown in Manhattan plots in Supplemental Figures 1 to 10 (see Supplemental Material available online, http://jas.fass.org). Table 4 is a summary of the number of SNP observed at various significance levels. The SNP effects, *P*-values, and minor allele frequencies for each SNP are presented in Supplemental Table 1. Table 5 summarizes SNP and chromosome information outputted from AWM analyses. Subsequently, and according to the PCIT algorithms, 1,555 important SNP of this study were linked by 113,873 significant correlations (or ~5% of the possible 2,416,470 total pairs). Table 1B contains results from deep-sequence transcriptome analyses of the hypothalamus of a prepubertal and postpubertal heifer, which includes number of reads (i.e., 36mers), number of reads aligned, and the number of annotations.

### *Network Analyses*

Significant correlations of genes observed by AWM-PCIT were visualized as a network, where 1,386 SNP were nodes and 5,132 strongest correlations ($|r| \geq 0.90$) were edges. This network was then filtered with genes queried from the hypothalamic-transcriptome resource as genes not common among these 2 types; these results were eliminated from further analyses. The remaining hypothalamic-influenced network contained 978 genes connected by 2,560 edges or predicted gene interactions. These results are illustrated in Figure 1A and B. The files formatted for Cytoscape containing these networks are available upon request. The gene networks, before and after hypothalamic-transcriptome filtering, were scale-free networks characterized by most nodes having a few connections and a few nodes having many connections (Figure 2). All further analyses considered the hypothalamic-influenced network, unless otherwise specified.

Pathway analysis performed with DAVID software suggested the network was enriched for genes of the axon guidance pathway ($P = 9.9 \times 10^{-6}$, after Bonferroni correction). Gene ontology analyses performed with GOrilla revealed enrichment for the terms: regulation of cellular localization ($P = 3.84 \times 10^{-7}$), regulation of secretion ($P$ = 5.19E-06), regulation of neurotransmitter secretion ($P$ = 1.4E-05), regulation of transport ($P$

**Table 3.** Correlations of measures of growth, ADG, and ultrasound measures of carcass in Brangus heifers. Above diagonal (bolded) are genetic correlations ± SE estimated via REML (shaded) and below diagonal are correlations estimated from genome-wide SNP effects used to construct an association weight matrix[1]

| r | $BW_{205}$ | $HT_{205}$ | $BW_{365}$ | $HT_{365}$ | ADG | LM area | BFT | IMF | FSC | HPG |
|---|---|---|---|---|---|---|---|---|---|---|
| $BW_{205}$ | | 0.81 ± 0.09 | 0.87 ± 0.06 | 0.76 ± 0.11 | 0.05 ± 0.23 | 0.72 ± 0.10 | 0.45 ± 0.18 | -0.11 ± 0.20 | 0.19 ± 0.39 | -0.28 ± 0.38 |
| $HT_{205}$ | 0.56 | | 0.70 ± 0.11 | 0.88 ± 0.07 | 0.02 ± 0.24 | 0.59 ± 0.14 | 0.60 ± 0.18 | 0.17 ± 0.21 | 0.23 ± 0.40 | -0.39 ± 0.39 |
| $BW_{365}$ | 0.65 | 0.34 | | 0.71 ± 0.11 | 0.54 ± 0.16 | 0.84 ± 0.06 | 0.64 ± 0.13 | -0.09 ± 0.19 | 0.21 ± 0.36 | -0.14 ± 0.35 |
| $HT_{365}$ | 0.43 | 0.66 | 0.55 | | 0.17 ± 0.23 | 0.55 ± 0.15 | 0.56 ± 0.18 | 0.05 ± 0.21 | 0.21 ± 0.40 | -0.23 ± 0.36 |
| ADG | 0.17 | 0.05 | 0.77 | 0.34 | | 0.46 ± 0.18 | 0.49 ± 0.20 | -0.02 ± 0.22 | 0.07 ± 0.39 | 0.20 ± 0.38 |
| LM Area | 0.52 | 0.22 | 0.74 | 0.31 | 0.53 | | 0.67 ± 0.12 | 0.1 ± 0.18 | 0.31 ± 0.33 | 0.17 ± 0.34 |
| BFT | 0.43 | 0.05 | 0.61 | 0.15 | 0.43 | 0.70 | | -0.08 ± 0.22 | 0.71 ± 0.34 | 0.27 ± 0.38 |
| IMF | 0.01 | -0.06 | 0.01 | -0.13 | 0.01 | 0.20 | 0.40 | | 0.10 ± 0.37 | 0.11 ± 0.35 |
| FSC | 0.16 | 0.11 | 0.14 | -0.01 | 0.12 | 0.24 | 0.15 | 0.02 | | 0.66 ± 0.40 |
| HPG | 0.17 | 0.05 | 0.10 | -0.05 | 0.08 | 0.18 | 0.11 | -0.01 | 0.73 | |

[1]$BW_{205\ \&\ 356}$ = BW at 205 and 365 d of age, respectively; $HT_{205\ \&\ 356}$ = hip height at 205 and 365 d of age, respectively; BFT = backfat; IMF = percent intramuscular fat; FSC = first service conception; HPG = heifer pregnancy

**Table 4.** Number of SNP observed by *P*-value categories from genome wide associations with measures of growth, ADG, and ultrasound measures of carcass, and fertility in Brangus heifers[1]

| | Traits | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *P*-value | $BW_{205}$ | $HT_{205}$ | $BW_{365}$ | $HT_{365}$ | ADG | LM Area | BFT | IMF | FSC | HPG |
| 0.05 | 1,742 | 1,655 | 2,189 | 1,568 | 1,853 | 2,116 | 2,189 | 2,244 | 1,589 | 1,509 |
| 0.01 | 362 | 284 | 487 | 294 | 364 | 463 | 506 | 557 | 284 | 232 |
| 0.001 | 29 | 22 | 53 | 21 | 45 | 65 | 75 | 85 | 20 | 16 |
| 0.0001 | 3 | 0 | 5 | 3 | 2 | 14 | 19 | 18 | 4 | 2 |
| 0.00001 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 7 | 0 | 0 |

[1]$BW_{205\ \&\ 356}$ = BW at 205 and 36 d of age, respectively; $HT_{205\ \&\ 356}$ = hip height at 205 and 365 d of age, respectively; BFT = backfat; IMF = percent intramuscular fat; FSC = first service conception; HPG = heifer pregnancy

= 3.3E-05), regulation of excitatory postsynaptic membrane potential (*P* = 7.07E-05), and regulation of membrane potential (*P* = 9.34E-05).

We applied network connectivity and centrality criteria to identify 59 clusters of highly interconnected genes and 56 hubs. Clusters were identified and ranked from 1 (high) to 59 (low), according to MCODE software scores, which reflected their complexity and centrality. Hubs and clusters are presented in Figure 1A and B. Note the reduced number of hubs in Figure 1B after the network was filtered with hypothalamic-transcriptome information.

### *Transcription Factors*

Transcription factors were considered important if they were hubs, meaning their regulatory role could impact the entire network. Five hubs were identified: *ZMAT3, RFX4, NR6A1, STAT6*, and *PLAGL1*. The names of these genes and their public database identification numbers are included in Supplemental Table 2 as are the names and identification numbers of all other genes described and discussed in these texts. The SNP that predicted these 5 TF were intragenic and their chromosome, SNP, and network connection information is included in Table 6. These hypothalamic-filtered networks can be visualized in Figure 1C. This figure also illustrates the connections predicted for 3 well-known positional candidate genes *IGF1, TSHR*, and *TGFβ3*.Connections represented the number of AWM-PCIT predicted targets (i.e., genes) for each TF. Predicted targets were included in regulatory sequence analysis, when matrix information derived from binding sites were available from the Genomatix database. This information was available for *NR6A1* (also known as *GCNF*)*, RFX,* STAT6, and *PLAGL1*, but no information was available for *ZMAT3*.

Table 6 summarizes the percentage of TF-gene target interactions within the network that could be validated by the presence of corresponding TFBS in RNA-Seq data from a prepubertal and postpubertal heifer. Table 7 lists the effect of each of the SNP predicting the 5 TF. There was a 12.6% improvement for the in silico validation of gene targets after filtering the network with hypothalamic-transcriptome information (i.e., 48.3 vs. 60.9%). Some important TF were predicted to interact directly with other TF in the network (Figure 1C). For example, *NR6A1* had predicted interactions with *TSHR* and *NEGR1*, and both of these were validated by the presence of 3 and 2 TFBS, respectively. Genes that possessed TFBS for *STAT6* were *KIF5A*, *MSRB3*, *PDZRN4*, and *R3HDM2*, respectively. For *RFX4*, it was *ANKS1B* and *HERC1*, whereas for *PLAGL1,* analyses revealed genes known as *DIAPH3*, *HERPUD1*, *RBL1*, *SFRS8*, *SLC12A3*, *SLC29A4*, *SRC*, *TPRG1*, and *ZMAT3*, respectively.

## DISCUSSION

The multitrait and polygenic approach described by Fortes et al. (2010a,b, 2011) was used to evaluate FSC data from Brangus heifers. First service conception was studied because it was a more evenly distributed binary trait than HPG (Table 2). This trait could be used as an indicator trait of early puberty as it reflects heifers that would have been responsive to estrous synchronization and AI at the start of their first breeding season, just as age of first corpus luteum (**CL**) was used in previous studies (Snelling et al., 2012). Several of these studies also involved *Bos indicus*-influenced heifers, which are known to have challenges achieving puberty early in life (Johnston et al., 2009; Riley et al., 2010; Luna-Nevarez et al., 2011).

In the current study, genes identified by GWAS and AWM-PCIT procedures were verified in the transcriptome of the hypothalamus and a filtered network was produced, which emphasized TF (Figure 1). In brief, SNP-chip data were used to identify 1,555 loci and related genes across the bovine genome and 978 of these genes were expressed in the hypothalamus. Network and TF analyses revealed gene hubs that mapped to chromosomes 1, 5, 9, and 11, respectively (Table 6). The ensuing discussion will focus on the relevance of these genes, especially *STAT6*, as well as the utility of studies involving multiple types of genomic and phenotypic data. The latter is particularly important as reproductive traits generally have low heritability estimates and data recording challenges (Gutierrez et al., 2002; Cammack et al., 2009; Johnston et al., 2009); thus, merging results

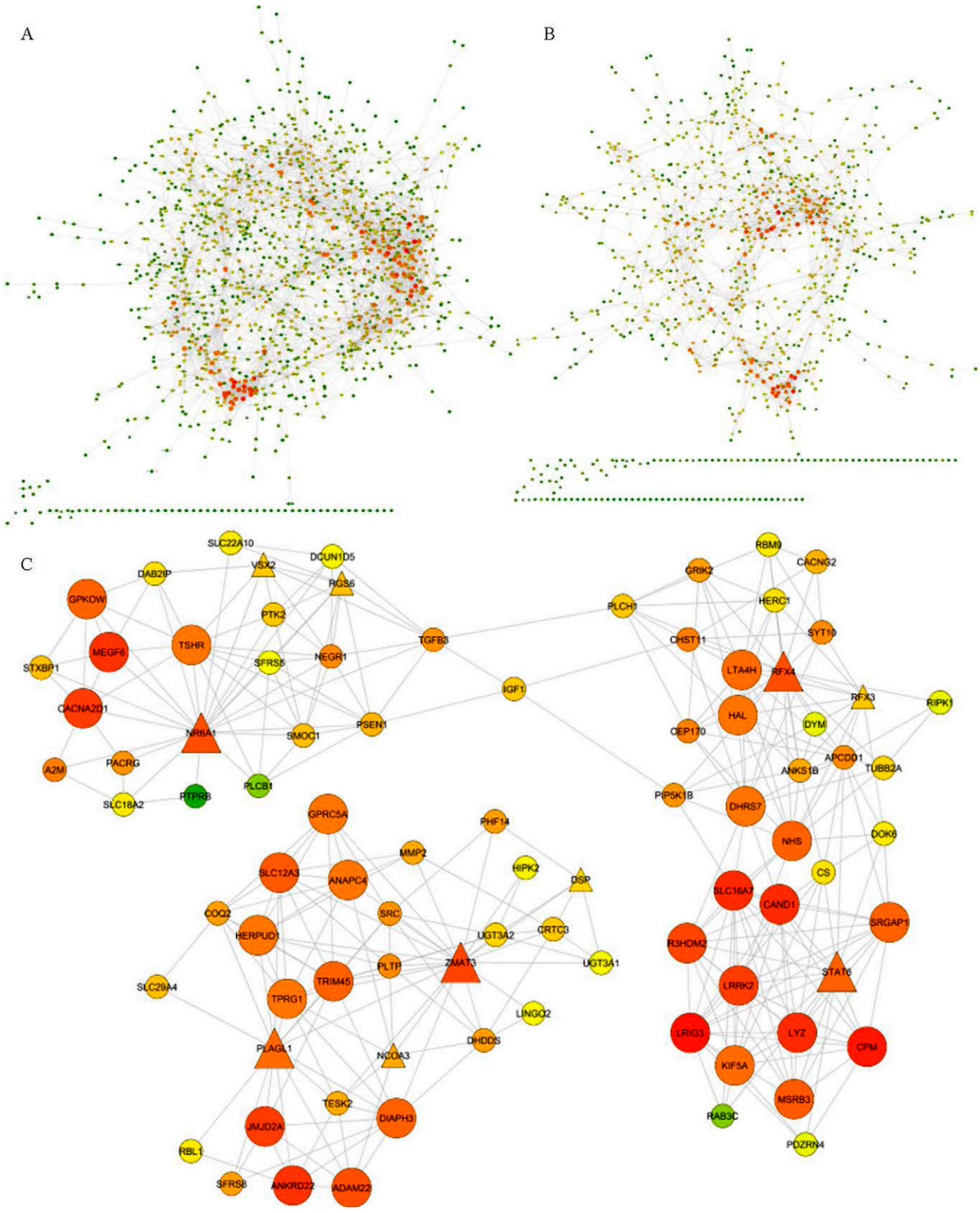


**Figure 1.** Associated weight matrix gene network A), hypothalamic-transcriptome filtered gene network B), and genes within networks containing transcription factors (TF) binding sites C). Size and color of nodes represent their number of connections, with bigger and brighter (red) nodes being the most connected. Triangles represent TF and circles represent all other genes. In C), 5 important TF (i.e., *ZMAT3*, *STAT6*, *RFX4*, *PLAGL1*, and *NR6A1*) and their predicted interactions with other genes can be visualized within the hypothalamic-transcriptome filtered gene network.

**Table 5.** Summary from associated weight matrix and partial correlation and information theory analyses of genome-wide SNP associations ($P < 0.05$) with measures of growth, carcass, and fertility in Brangus heifers

| Chromosome | No. SNP ≤2500 bp of a gene | No. SNP >2,500 bp and <1.5 Mb of a gene | No. SNP ≥1.5 Mb from a gene | Total No. SNP |
|---|---|---|---|---|
| 1 | 76 | 4 | 2 | 82 |
| 2 | 65 | 4 | | 69 |
| 3 | 95 | 3 | | 98 |
| 4 | 60 | | 1 | 61 |
| 5 | 113 | 9 | | 122 |
| 6 | 61 | 2 | | 63 |
| 7 | 76 | 13 | | 89 |
| 8 | 67 | 3 | 6 | 76 |
| 9 | 48 | 4 | 2 | 54 |
| 10 | 64 | 2 | | 66 |
| 11 | 46 | | | 46 |
| 12 | 31 | 1 | 3 | 35 |
| 13 | 70 | | | 70 |
| 14 | 30 | 1 | | 31 |
| 15 | 59 | 3 | 2 | 64 |
| 16 | 64 | 1 | 3 | 68 |
| 17 | 20 | | | 20 |
| 18 | 44 | | | 44 |
| 19 | 61 | 2 | | 63 |
| 20 | 52 | 3 | 2 | 57 |
| 21 | 38 | 1 | | 39 |
| 22 | 40 | | | 40 |
| 23 | 20 | | | 20 |
| 24 | 20 | | | 20 |
| 25 | 24 | | | 24 |
| 26 | 26 | | 2 | 28 |
| 27 | 24 | | 3 | 27 |
| 28 | 27 | | | 27 |
| 29 | 40 | 1 | | 41 |
| x | 11 | | | 11 |
| Totals | 1,472 | 57 | 26 | 1,555 |

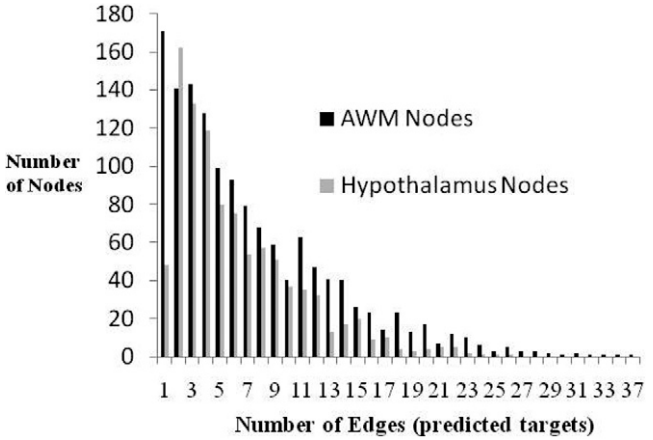


**Figure 2.** Network connectivity expressed by the number of edges (X axis) per node (Y axis). Columns in black represent data from 1,555 selected SNP from an associated weight matrix (AWM); columns in gray are from the 978 genes expressed in the hypothalamic-transcriptome.

from various types of genomic data may have utility in gene discovery, as well as genetic prediction.

Construction of gene networks and pathways allow visualization of a large number of genes and their interactions for complex traits (Lee et al., 2010; Marbach et al., 2010; Butte et al., 2011). The network constructed in this study had a scale-free topology, which represented the nonrandom nature of the network. In a random network, most genes have similar number of connections, which is challenging to identify highly connected clusters or hubs (Barbási and Oltvai, 2004). The network of the current study was congruent, with most biological networks that are scale free as we identified 54 clusters of highly connected genes and 42 hubs, including 5 hubs that were annotated as TF.

The *STAT6* gene was detected as a network hub in the fertility data from Brangus heifers of this study. Previously, *STAT6* was associated with age at first CL in Australian Brahman and tropical composite heifers (Fortes et al., 2010a, 2011). Failure to identify other genes in common among these studies may be for multiple reasons. For example, heifer fertility phenotypes were derived from different methodologies (i.e., ovarian ultrasound to detect CL vs. conception from an estrous synchronization and AI protocol) or limitation of BovineSNP50 data or both to accurately capture the genomic architecture of the populations studied (Gibbs et al., 2009; Matukumalli et al., 2009; Bolormaa et al., 2011b). Future studies using genome-wide surveys with denser chromosome coverage may yield results that are more applicable across breeds, populations, or studies, or a combination of all 3. We anticipate that the approaches described herein will gain importance due to their ability to reveal pathways and networks associated with variation in traits. It is also important to note that the networks described herein were scale free, so the transcription factors detected were hubs with many connections.

Thanks to the advent of whole-genome bovine DNA and RNA analyses coupled with bioinformatics tools, we can readily generate gene networks as presented in Figure 1. Via assessment of gene expression, transcriptome resources confirm genes identified by methodologies using GWAS. A similar approach was used to identify the genetic drivers of pigmentation in Merino sheep (Garcia-Gamez et al., 2011). Specifically, 49,034 SNP genotypes were combined with gene expression data from microarray analyses (i.e., 11,689 probes) of 5 skin-tissue types. In the current study, GWAS results from 34,894 SNP genotypes were combined with 22,995 annotations/genes in the hypothalamus that were identified by RNA-Seq, which is a procedure that improved quantitative assess-

**Table 6.** Results from gene network and transcriptions factor (TF) analyses of genomic and multitrait information of Brangus heifers. Predicted targets are number of interacting genes derived from network analyses and validated targets are number of genes with corresponding transcription factor binding site sequences

| | | | | Before hypothalamic-transcriptome filtering | | | After hypothalamic-transcriptome filtering | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Chr[1] | TF | SNP name[2] | SNP Position (Mb)[2] | No. Predicted Targets | No. Validated Targets | % validated | No. Predicted Targets | No. Validated Targets | % validated | $P^3$ |
| 5 | *STAT6* | Hapmap30258-BTA-143119 | 60.84 (56.67) | 22 | 6 | 27.3 | 15 | 6 | 40.0 | 0.07 |
| 5 | *RFX4* | Hapmap52789-rs29018750 | 75.06 (70.26) | 24 | 9 | 37.5 | 19 | 9 | 47.4 | 0.07 |
| 9 | *PLAGL1* | ARS-BFGL-NGS-99576 | 84.18 (82.42) | 21 | 13 | 61.9 | 15 | 12 | 80.0 | 0.01 |
| 11 | *NR6A1* | Hapmap45640-BTA-113575 | 99.07 (95.64) | 24 | 16 | 66.7 | 21 | 16 | 76.2 | 0.03 |
| | | | Totals | 91 | 44 | 48.34 | 70 | 43 | 60.89 | 0.000002 |
| 1 | *ZMAT3*[4] | BTB-01349174 | 90.36 (135.38) | | | | | | | |

[1]Chr = chromosome.

[2]SNP intergenic for the gene described as a TF and positions based on assemblies of *Bos taurus* version 4.0 and University of Maryland version 3.1 positions.

[3]*P* determined with hypergometric comparison of number validated relative number predicted targets.

[4]*ZMAT3* was not present in Genomatix database.

ment of gene expression relative to microarray (Mardis, 2008; Mortazavi et al., 2008; Wang et al., 2009a).

It is important to note that the transcriptome data of the current study were from deep sequencing. This approach of sequencing a sample many times yields multiple copies of the code and enhances the ability to detect polymorphisms and gene expression (Van Tassell et al., 2008; Harhay et al., 2010; Mamo et al., 2011). The rationale for deep sequencing RNA from a prepubertal and postpubertal heifer was to pool the results from the 2 heifers and produce a comprehensive list of genes expressed in the hypothalamus. Harhay et al. (2010) and Ravasi et al. (2010) described these lists as a gene atlas. In the current study, this list of genes (i.e., gene atlas) was used to filter the network produced by GWAS-AWM-PCIT methodology as to understand genes expressed in a key tissue of reproduction, hypothalamus.

Network results from analyses of Brangus heifer data and the reports by Fortes et al. (2010a, 2011) yielded a plethora of gene information. To mine this information to reveal genetic drivers of traits or physiological mechanisms, procedures such as filtering or screening with TF analyses are needed. Previous reports of hypothalamic networks in model species were important in deciding to study TF (Roth et al., 2007; Ojeda et al., 2010ab; Mueller et al., 2011). However, there are many approaches that can be used to filter a network. Perhaps, the development of neuropeptide or proteomic profiling, or both can expand our efforts to include protein information with gene expression analyses (Nomura et al., 2010; Colgrave et al., 2011; Dudley et al., 2011).

Network analyses revealed 5 TF as hubs: *ZMAT3*, *STAT6*, *RFX4*, *PLAG1*, and *NR6A1*. The *STAT6* gene maps to chromosome 5, where there are several other genes known to be components of the GH-IGF axis (i.e., *IGF1,IGFBP6, STAT*2, and *SOCS*2; Farber et al., 2006). The signal transducer and activator of transcription (**STAT**), and suppressor of cytokine signaling (**SOCS**) proteins are involved in cell signaling cascades subsequent to proteins or hormones, or both, such as GH, leptin*, IL4*, and *IFNγ*, binding their receptors (Schindler and Plumlee, 2008; Ahmed and Farquharson, 2010; Ricardo-Gonzalez et al., 2011). Polymorphisms within the STAT genes were associated with growth, carcass, and fertility traits in studies of multibreed populations of cattle, which involved Angus × Brahman crosses (Rincon et al., 2009; Luna-Nevarez et al., 2011). It should be noted that the intragenic *STAT6* SNP on

**Table 7.** The SNP effect within the genes *ZMAT3*, *STAT6*, *RFX4*, *PLAG1*, and *NR6A1* identified, using genomic and multitrait information of Brangus heifers in network and transcription factor analyses (TF)[1]

| TF | $BW_{205}$ | $HT_{205}$ | $BW_{365}$ | $HT_{365}$ | ADG | LM area | BFT | IMF | FSC | HPG |
|---|---|---|---|---|---|---|---|---|---|---|
| *ZMAT3* | –3.54 | –0.83 | –3.82 | –0.81 | 0.006 | –0.13 | –0.01 | 0.06 | 0.09* | 0.04 |
| *STAT6* | –0.91 | 0.42 | 0.44 | 0.44 | 0.004 | –0.44 | –0.02* | –0.14** | –0.01*** | –0.02 |
| *RFX4* | 0.38 | –0.20 | 1.74 | –0.19 | 0.009 | 0.37 | 0.02* | 0.12** | –0.02 | –0.01 |
| *PLAGL1* | 0.93 | 0.65* | 1.44 | 0.70* | –0.002 | 0.37 | 0.01 | –0.01 | –0.06 | –0.05* |
| *NR6A1* | –0.91 | –0.13 | –4.00 | –0.33 | –0.010 | –0.07 | –0.02* | –0.10* | 0.04 | 0.04 |

[1]$BW_{205\ \&\ 356}$ = BW at 205 and 365 d of age, respectively; $HT_{205\ \&\ 356}$ = hip height at 205 and 365 d of age, respectively; BFT = backfat; IMF = percent intramuscular fat; FSC = first service conception; HPG = heifer pregnancy. Units of traits described in Table 2.

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

BovineSNP50 is within intron 2 and within a few kb of the ETH10 microsatellite adjacent to exon 1, which is used for parentage verification in cattle (Matukumalli et al., 2009; DeAtley et al., 2011).

Chromosome 5 is very gene dense and has been reported to harbor QTL associated with growth and fertility phenotypes (Bolormaa et al., 2011a,b,c; Hawken et al., 2012). The discussion in these manuscripts described *IGF1* as a positional candidate gene under these QTL. Note *IGF1* within the networks in Figure 1C. Both *IGF1* and leptin have been studied intensively for their physiological relationship to puberty in *Bos indicus*-influenced heifers (Garcia et al., 2002; Shirley et al., 2006; Johnston et al., 2009). Therefore, the observations in the current study of genes relevant to adiposity and puberty should have been expected, as there were also genetic associations between measures of backfat and FSC and HPG (Table 3).

Two other transcription factors that had network connections with *STAT6* were *NR6A1* and *RFX4*. It is very interesting that *NR6A1* was also reported to be involved in transcriptional regulation of hypocretin/orexin (Tanaka et al., 2010), which is an appetite-regulating peptide expressed in the hypothalamus (Anukulkitch et al., 2010; Lopaschuk et al., 2010). The hypothalamus is a neural tissue that regulates many events, such as appetite, metabolic rate, and reproduction. Previous physiology study of Brangus heifers suggested there is a relationship between feed efficiency and age at puberty among certain sires (Shirley et al, 2006). Thus, it was understandable that *NR6A1*and other well-known proteins involved in growth and metabolism were identified by our network approach.

The network reported herein was enriched for genes of the axon guidance pathway. Axon guidance processes influence pulsatile release of LHRH, which is essential for attaining puberty (Rodrigues et al., 2002; Clarkson and Herbison, 2006; Bliss et al., 2010). Thus, the 5 TF hubs revealed in this study are expected to exert an influence on the reproductive axis in heifers via genes involved in axon guidance. For example, *ZMAT3* has a protein-protein interaction with *ESR1* via an interaction with the oncogene *p53* (Wang et al., 2009b; Ravasi et al., 2010; Vilborg et al., 2010). These reports in model species are of interest as a binding protein of *p53* and the estrogen receptor (*ESRRG*) were revealed as candidate genes in the analyses of heifers from the tropics (Fortes et al., 2010a).

There is a growing body of literature describing relationships of TF, such as *NR6A1* and *PLAG1*, and anthropometric measures in livestock (Soma et al., 2010; Karim et al., 2011; Mikawa et al., 2011). In the current study, a gene known as *PLAGL1* was detected in TF analyses and it was found to have connection with *ZMAT3* (Figure 1C). Pleomorphic adenoma genes have been implicated in regulation of *IGF2* and IGF receptors, as well as genes of the *TGFβ* family (Van Dyck et al., 2007; Elledge, 2009; Furuse et al., 2010). Study of the bovine *PLAG1* gene by Karim et al. (2011) revealed the challenges of clarifying functional mutations in the many candidate genes, which may underlie a QTL. Nonetheless, networking procedures are advancing so that gene expression dynamics can be observed among samples collected from animals of varied physiological states and/or treatments (Prentice et al., 2011).

This study of growing Brangus heifers confirms the complexity of fertility traits, such as FSC. Positional candidate genes (i.e., *ZMAT3*, *STAT6*, *RFX4*, *PLAG1*, and *NR6A1*) were identified from a systems biology approach, using multiple genomic resources (i.e., GWAS, RNA-Seq, and TF). These findings also provide insight into the hypothalamic mechanisms regulating fecundity of heifers. It should be noted that *STAT6* was detected as hub in network data from Brangus heifers reared in the United States, as well as Brahman and tropical composite heifers of Australia (Fortes et al., 2010a, 2011).

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