Global differential gene expression in the pituitary gland and the ovaries of pre- and postpubertal Brahman heifers[1]

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ABSTRACT: To understand genes, pathways, and networks related to puberty, we characterized the transcriptome of two tissues: the pituitary gland and ovaries. Samples were harvested from pre- and postpubertal Brahman heifers (same age group). Brahman heifers (*Bos indicus*) are older at puberty compared with *Bos taurus*, a productivity issue. With RNA sequencing, we identified differentially expressed (DEx) genes and important transcription factors (TF) and predicted coexpression networks. The number of DEx genes detected in the pituitary gland was 284 ($P < 0.05$), and *VWC2L* was the most DEx gene (fold change = 4.12, $P = 0.01$). The gene *VWC2L* promotes bone mineralization through transforming growth factor-β (TGFβ) signaling. Further studies of the link between bone mineralization and puberty could target *VWC2L*. In ovaries, 3,871 genes were DEx ($P < 0.05$). Four highly DEx genes were noteworthy for their function: *SLC6A13* (a γ-aminobutyric acid [GABA] transporter), *OXT* (*oxytocin*), and *NPY* (*neuropeptide Y*) and its receptor *NPY2R*. These genes had higher ovarian expression in postpubertal heifers. The GABA and its receptors and transporters were expressed in the ovaries of many mammals, suggesting a role for this pathway beyond the brain. The *OXT* pathway has been known to influence the timing of puberty in rats, via modulation of GnRH. The effects of *NPY* at the hypothalamus, pituitary gland, and ovaries have been documented. *Neuropeptide Y* and its receptors are known factors in the release of GnRH, similar to *OXT* and GABA, although their roles in ovarian tissue are less clear. Pathways previously related to puberty such as TGFβ signaling ($P = 6.71 \times 10^{-5}$), Wnt signaling ($P = 4.1 \times 10^{-2}$), and peroxisome proliferator-activated receptor (PPAR) signaling ($P = 4.84 \times 10^{-2}$) were enriched in our data set. Seven genes were identified as key TF in both tissues: *HIC2*, *ZIC4*, *ZNF219*, *ZSCAN26*, *LHX1*, *OLIG1*, and a novel gene. An ovarian subnetwork created with TF and significant ovarian DEx genes revealed five zinc fingers as regulators: *ZNF507*, *ZNF12*, *ZNF512*, *ZNF184*, and *ZNF432*. Recent work of hypothalamic gene expression also pointed to zinc fingers as TF for bovine puberty. Although some zinc fingers may be ubiquitously expressed, the identification of DEx genes in common across tissues points to key regulators of puberty. The hypothalamus and pituitary gland had eight DEx genes in common. The hypothalamus and ovaries had 89 DEx genes in common. The pituitary gland and ovaries had 48 DEx genes in common. Our study confirmed the complexity of puberty and suggested further investigation on genes that code zinc fingers.

Key words: *Bos indicus*, gene expression, gene networks, ovarian tissue, pituitary gland, transcription factors, zinc fingers

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INTRODUCTION

Puberty is a process initiated by the altered biphasic release of GnRH. The release of GnRH stimulates the secretion of LH and FSH from the pituitary gland. Via the bloodstream, pituitary gland hormones bind to receptors in the gonads and stimulate ovulation and the production of the gonadal steroid hormones including progesterone, estrogen, activins, and inhibins (Day et al., 1984, 1987; Schillo et al., 1992; Gasser et al., 2006; Bliss et al., 2010). Gonadal steroids such as estrogen and progesterone are released into the bloodstream and then inhibit or stimulate the release of GnRH in a cyclic manner related to ovarian dynamics during the estrus cycle (Bliss et al., 2010); this is a complex feedback loop that influences the progression of puberty. Estrogen also modulates LH and FSH release via its receptors (Gharib et al., 1987; Shupnik et al., 1988, 1989), and both estrogen receptor α and β (ERα and ERβ) are observed in the hypothalamus, pituitary gland, and ovaries (Hatoya et al., 2003). Although these events are similar in *Bos indicus* and *Bos taurus* cattle, they are initiated later in *B. indicus*. Puberty in *B. indicus* included an increase in LH release whereas FSH release did not change (Nogueira et al., 2003), and these hormonal patterns were similar in *B. taurus* breeds (Evans et al., 1994). Furthermore, study of 17β-estradiol negative feedback on LH release noted a decreased feedback at an older age in *B. indicus* heifers than that of *B. taurus* heifers (Rodrigues et al., 2002). The *B. indicus* cattle are better adapted to tropical regions, and the difference of region adaptation seems to have resulted in reproductive function differences in the two breeds (Mezzadra et al., 1993; Wolfenson et al., 2000; Nogueira, 2004). However, knowledge of mechanisms controlling the onset of puberty in *B. indicus* is still limited. Ribonucleic acid sequencing is an emerging technology to analyze global differential expression (Mortazavi et al., 2008; Wang et al., 2009; Huang and Khatib, 2010). We used RNA sequencing of the pituitary gland and ovaries to identify differentially expressed (**DEx**) genes and pathways related to puberty in age- and weight-matched Brahman heifers. The difference of gene expression before and after pubertal development between Brangus heifers (Cánovas et al., 2014a) and Brahman heifers was also reported.

MATERIALS AND METHODS

Animals and Samples

Heifers were managed, handled, and euthanized per approval of the Animal Ethics Committee of The University of Queensland, Production and Companion Animal group (certificate number QAAFI/279/12). The 12 Brahman heifers selected in this study were from the same age group and represented commercial animals and were sourced as weanlings (approximately 6 mo of age) in October 2012 from two breeders (Fortes et al., 2016). On arrival, heifers weighed between 176 and 244 kg. Heifers were managed similarly and fed a pasture-based diet at the Gatton Campus facilities of the University of Queensland (Gatton, Australia).

Observation of pubertal development was performed every fortnight from October 2012 to May 2013. Pubertal status was defined by the observation of the first corpus luteum (**CL**) using ultrasound (Johnston et al., 2009). Six postpubertal heifers were euthanized on the luteal phase of their second estrus cycle, confirmed by the presence of CL. Heifers were euthanized 23 d, on average, after observation of the first CL. Prepubertal heifers were randomly selected from the group that had never ovulated and were paired with postpubertal heifers for the slaughter days. The plasma progesterone concentration was measured to confirm functional CL in postpubertal heifers (average 2.0 ± 0.7 ng/mL). The average concentration of progesterone in prepubertal heifers was lower, 0.4 ± 0.2 ng/mL. Plasma progesterone concentrations were measured by RIA at the Animal Endocrinology Laboratory of the University of Queensland (Brisbane, Australia). Progesterone concentrations in hexane extracts of the plasma samples were measured by RIA as described by Curlewis et al. (1985), except that the progesterone antiserum used was C-9817 (Bioquest Ltd., North Ryde, NSW, Australia). Extraction efficiency was 75% and the values reported herein were not corrected for these losses. The sensitivity of the assay was 0.1 ng/mL and the intra- and interassay CV was 5.0%.

Body weight (kg), condition scores (5-point scale), and ADG were also measured before tissue harvesting. Body weight averages and SD for pre- versus postpubertal heifers were 338 kg (SD 54.17) and 363 kg (SD 38.62), respectively. Condition score (5-point scale [1–5]) averages and SD for pre- and postpubertal heifers were 3.5 (SD 0.44) and 3.75 (SD 0.41), respectively. In pre- and postpubertal heifers, ADG and SD were 0.76 kg/d (SD 0.1) and 0.89 kg/d (SD 0.1), respectively. No significant difference in BW ($P = 0.38$), condition scores ($P = 0.18$), and ADG ($P = 0.16$) between pre- and postpubertal animals was observed at euthanasia (Fortes et al., 2016).

Tissue harvesting was executed as fast as possible (within 20 min after euthanasia) to preserve RNA quality. The pituitary gland and both ovaries from each heifer were snap-frozen in liquid nitrogen after harvesting and then kept at −80°C until RNA extraction. In total, 12 pituitary glands (including anterior and posterior pituitary gland tissues) were separately processed for RNA extraction and sequencing. A total of 24 ovaries were separately processed for extraction

and sequencing, with a record for CL presence in left or right ovary maintained in the postpubertal heifers. No evidence for large preovulatory follicles was observed on euthanasia day in either pre- or postpubertal heifers.

Ribonucleic Acid Extraction and Sequencing

Prior to RNA extraction, 12 pituitary gland and 24 ovarian tissues were pulverized under liquid nitrogen and homogenized to form a uniform sample representative of the whole organ. Total RNA was isolated from fragmented frozen tissues (approximately 25 mg) using a combination of RNeasy and TRIzol methods. In brief, tissue samples were homogenized in 1 mL of TRIzol reagent (Sigma-Aldrich Pty. Ltd., Sydney, NSW, Australia) with a QIAshredder homogenizer (QIAGEN Pty. Ltd., Melbourne, VIC, Australia). Then, 400 μL chloroform (Sigma-Aldrich Pty. Ltd.) was added and the samples were vigorously mixed by hand and allowed to stand 5 min at room temperature followed by centrifugation at 12,000 × *g* for 20 min at 4°C. The upper aqueous layer containing RNA was transferred to a new tube and a 1.5 mL volume of ethanol (100%; Chem-Supply Pty. Ltd., Adelaide, SA, Australia) was added and mixed. The mixture was transferred to an RNeasy mini column (QIAGEN Pty. Ltd.) and RNA purification followed the kit's protocol (QIAGEN Pty. Ltd.).

The quality of the total RNA was evaluated using the RNA integrity number value in an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Values for RNA integrity number ranged from 7 to 9, indicating good quality of RNA samples: intact 28S and 18S rRNA subunit integrity and no traces of DNA detected. High quality RNA samples were sent to the University of California, Davis, for library preparation and sequencing. A TruSeq RNA sample preparation kit (Illumina Inc., San Diego, CA) was used to first purify the RNA; mRNA purification used poly T oligo attached magnetic beads. Purified mRNA was then fragmented and converted to cDNA, which was double stranded, ligated to adapters, and amplified with PCR to create the libraries (all performed per kit protocol, following manufacturer's instructions). Libraries were sequenced with an Illumina HiSeq analyzer (Illumina Inc., San Diego, CA). The bovine reference genome, also known as the UMD3.1 assembly (release 77; ftp://ftp.ensembl.org/pub/release-77/genbank/bos_taurus; accessed May 15, 2015), was used as the reference genome for sequence assembly, which was performed using the CLC workbench software (CLC bio, Aarhus, Denmark). This software was also used for quality control of sequence data and calculation of reads per kilobase per million mapped reads (**RPKM**) per gene (Mortazavi et al., 2008; Cánovas et al., 2013). The CLC workbench software assesses sequence quality indicators based on the protocols of the FastQC project (http://www.bioinformatics.babraham.ac.uk/projects/fastqc; accessed May 20, 2015). Quality was measured, taking into account sequence-read lengths and base coverage, nucleotide contributions and base ambiguities, quality scores as emitted by the base caller, and over-represented sequences (Cánovas et al., 2014b). All the samples analyzed passed all the quality control parameters having the same length (100 bp); 100% coverage in all bases; 25% of A, T, G, and C nucleotide contributions; 50% GC on base content; and less than 0.1% over-represented sequences, indicating good quality of sequencing outputs. Only transcripts with RPKM ≥ 0.2 in at least 1 tissue were considered expressed genes; otherwise, they were discarded from further analyses (Wickramasinghe et al., 2012; Cánovas et al., 2014a). Uniform RPKM values across tissues may serve to identify ubiquitous genes or tissue-specific genes, but these analyses should be performed across all studied tissues and is beyond the current study, which is focused on pituitary gland and ovarian expression.

Differential Gene Expression

Differentially expressed genes in the pituitary gland and ovaries of post- versus prepubertal heifers were identified based on normalized RPKM values. We fitted a mixed model to the base-2 log transformed RPKM values as previously detailed (Reverter et al., 2004; Cánovas et al., 2014a; Fortes et al., 2016). In this mixed model, the library was fitted as a fixed effect, the main effect of gene was fitted as a random effect, and tissue × gene × animal was fitted as a random interaction. Our study is part of a larger experiment where five tissues were sampled per animal (hypothalamus, pituitary gland, liver, ovary, and uterus). The library effect was not confounded with animal as there were 5 libraries per animal (1 for each tissue). The VCE6 software (ftp://ftp.tzv.fal.de/pub/vce6; accessed June 15, 2015) was used to estimate variance components and to obtain solutions of the mixed model. For each gene, the average gene expression in post- versus prepubertal heifers was estimated from the normalized expression values. A *t* test was used to test the hypothesis that the differential expression in post- versus prepubertal heifers was significant, and the *P*-value of <0.05 was used as the threshold to determine DEx genes. This nominal *P*-value of <0.05 threshold was used in context with the strict normalization performed and the subsequent analyses, which used the DEx genes for further scrutiny.

Key Regulators and Gene Network Prediction

To determine the regulators in our data set, the AnimalTFDB bovine database (http://bioinfo.life.hust.edu.cn/AnimalTFDB/download_index?tr=Bos_taurus; accessed June 20, 2015) was used. This database comprises classification and annotation of transcription factors (**TF**), chromatin remodeling factors, and transcription cofactors. A regulatory impact factor (**RIF**) metric was used to identify key regulators among TF expressed in the pituitary gland and ovaries (Hudson et al., 2009; Reverter et al., 2010). The metric RIF was explored in its two measures, RIF1 and RIF2, calculated from the number of DEx genes and the predicted interactions between TF and target DEx genes (Reverter et al., 2010). Generally, RIF1 captures those TF that showed a large differential connectivity to highly abundant DEx genes (distinct network connections in pre- and postpubertal heifers' data). Meanwhile, RIF2 captures TF showing evidence as predictors of change in abundance of DEx genes. For comparisons between RIF1 and RIF2 and across data sets, RIF measures were transformed to a z-score by subtracting the mean and dividing by the SD. Using a *P*-value cutoff of 0.05, a TF was considered a top-ranking regulator if either of the two RIF scores were higher than 1.96 SD units.

The partial correlation and information theory (**PCIT**) algorithm was used to detect the associations between DEx genes and all expressed TF in a coexpression gene network (Reverter and Chan, 2008). In brief, PCIT explores all correlations between possible triplets of genes before estimating informative correlation between gene pairs. A partial correlation between two genes is the correlation between this particular pair of genes that is independent of a third gene. Using Cytoscape (Shannon et al., 2003), coexpression networks that combined pre- and postpubertal data to predict gene interactions within each of studied tissues, one for the pituitary gland and one for ovaries, were visualized and analyzed. In addition, we explored a subnetwork comprising the significant coexpression correlations between top-ranking TF and highly DEx genes (absolute fold change $|\mathbf{FC}| > 1$, $P \leq 0.01$) in ovaries.

Functional Enrichment Analyses

Gene ontology (**GO**) enrichment and pathways analysis were performed with three types of gene lists: 1) DEx genes from the pituitary gland or ovaries, 2) top-ranking TF from the pituitary gland or ovaries, and 3) genes and TF that formed the predicted networks for the pituitary gland or ovaries. These lists of genes were used as target gene lists (one at a time) to compare with a background gene list formed by all genes expressed in these tissues. The annotation terms enriched for these lists of genes were identified using a DAVID functional annotation chart (Huang et al., 2009). The functional annotation chart identified and reported the most relevant biological terms (over-represented) associated with these gene lists, reporting an enrichment *P*-value for each annotation term. Significant enrichment results after Benjamini–Hochberg correction for multiple testing are reported.

Comparative Analyses with Brangus Heifers

To provide further evidence for DEx genes reported in this experiment, we compared our findings with those from Cánovas et al. (2014a). The paper from Cánovas et al. (2014a) described a similar pre- and postpubertal Brangus heifers experiment. Noteworthy differences between their work and the work reported here are 1) the cattle breed and 2) the fact that in the Cánovas et al. (2014a) paper, the prepubertal heifers were younger and lighter than the postpubertal ones as opposed to being age- and weighted-matched controls. Despite differences, finding DEx genes in common between the two experiments was an aim for the current work, and this is described in our results. We used a nominal *P*-value < 0.05 to identify genes considered significant in each experiment and then compared the resulting lists to mine genes in common, per tissue. Genes in common between the two experiments were observed for their similar or divergent DEx pattern (up- or downregulation in postpubertal heifers).

RESULTS

Seven genes formed the overlap between the pituitary gland and ovaries for top-ranking TF, according to the RIF metric: *HIC2*, *ZIC4*, *ZNF219*, *ZSCAN26*, *LHX1*, *OLIG1*, and a novel gene (Table 1). There were 48 genes that were DEx in both tissues, meaning that the majority of the DEx results were tissue specific. Tissue-specific results are described separately in the next sessions to facilitate interpretation of results.

Overall, we found 16,978 genes expressed (RPKM > 0.2 on average in at least one tissue) in our samples, and therefore, by chance alone, 849 genes could be detected as significant for a *P*-value lower than 0.05, a nominal threshold.

Pituitary Gland Results

The number of DEx genes detected in the pituitary gland tissues was 284 (Supplementary Table S1; see the online version of the article at http://journalofanimalscience.org). In the pituitary gland, 165 genes were downregulated and 119 genes were upregulated after

Table 1. Transcription factors deemed important regulators according to the regulatory impact factor (RIF) metric in both pituitary and ovarian tissues (RIF > 1.96; $P < 0.05$)

ENSB tag[1]	Gene[2]	Chr.[3]	RIF1_Pit[4]	RIF2_Pit[4]	RIF1_Ova[4]	RIF2_Ova[4]	Description[5]
ENSBTAG00000005951	HIC2	17	−2.65	0.22	−2.35	−0.22	Hypermethylated in cancer 2
ENSBTAG00000014749	ZIC4	1	−1.97	−1.10	2.08	0.54	Zic family member 4
ENSBTAG00000021008	ZNF219	10	0.52	2.06	−0.18	−2.16	Zinc finger protein 219
ENSBTAG00000031869	ZSCAN26	23	2.11	1.35	−2.17	0.34	Zinc finger and SCAN domain containing 26
ENSBTAG00000033562	LHX1	19	−2.25	−0.09	2.06	0.53	Bos taurus LIM homeobox 1
ENSBTAG00000037547	OLIG1	1	−2.23	0.07	2.06	0.54	Bos taurus oligodendrocyte transcription factor 1
ENSBTAG00000040442	Novel gene	18	2.23	0.55	−2.64	1.51	Uncharacterized protein

[1]ENSB tag = Ensembl gene identifier according to http://uswest.ensembl.org/index.html (accessed 24 May 2016).

[2]Gene symbol related to the ENSB tag.

[3]Chr. = chromosome of gene location in the bovine.

[4]RIF1 = regulatory impact factor 1; RIF2 = regulatory impact factor 2; "Pit" indicates pituitary gland tissue results and "Ova" indicates ovarian tissue results.

[5]Brief description about the gene associated with each ENSB tag.

puberty. Of these 284 DEx genes, 55 had a $|FC| \geq 2$ (either up or down). Among the pituitary gland DEx genes, there were 12 TF, three transcription cofactors, and one chromatin remodeling factor observed. Genes highly DEx ($|FC| \geq 3$, $P \leq 0.01$) in the pituitary are shown in Fig. 1 and Table 2. For example, *VWC2L* (part of the transforming growth factor-β [**TGF**β] pathway) was among the DEx genes with higher FC, and its potential role in puberty is further explored in the discussion section.

Enrichment analyses of GO terms were performed using the 284 DEx genes in the pituitary gland as the target gene list and the genes expressed in both the pituitary gland and ovaries (16,978 genes) as a background list. In the pituitary gland, no GO terms or pathways were enriched after correction for multiple testing. Only 1 keyword term approached significance, "secreted," with 22 genes associated to this term ($P = 0.06$; keywords were defined by the SwissProt/Uniprot [http://www.uniprot.org/; accessed 1 June 2016] and Protein Information Resource [3 June 2016]; Supplementary Table S2 [see the online version of the article at http://journalofanimalscience.org]). The enrichment for the term "secreted" should not be surprising considering that this is an active gland.

Using the RIF metrics, we identified 119 significant top-ranking TF ($P < 0.05$) in the pituitary gland (Supplementary Table S3; see the online version of the article at http://journalofanimalscience.org). Of these 119 TF, 27 genes coded for TF of the zinc finger family. The TF *GATAD2B*, *BARHL1*, *SP5*, and *ZNF8* had the highest scores in RIF1 (>3 SD units). All of these high-scoring TF had lower expression in postpubertal heifers ($P < 0.05$). Pathway analysis for 119 TF identified in the pituitary gland suggested these TF were enriched for seven pathways (Supplementary Table S4; see the online version of the article at http://journalofanimalscience.org), all related to cancer, including endometrial cancer and leukemia.

Coexpression networks were predicted with PCIT for the pituitary gland using the expression values for DEx genes and all expressed TF in our samples (1,085 TF). The predicted network from pituitary gland results had 7,403 connections for 1,284 genes (Fig. 2A). In this network, *LIM homeobox 1* (*LHX1*), *Forkhead box F1* (*FOXF1*), and a novel gene (uncharacterized protein ENSBTAG00000033642) were the only 3 genes that were simultaneously top-ranking TF (RIF score > 1.96) and DEx ($P < 0.05$). Notably, *LHX1* was identified as one of the top-ranking TF in the ovaries as well (RIF1 = 2.05).

Tools in the DAVID functional annotation chart (Huang et al., 2009) were used to identify enriched pathways from the genes in the predicted pituitary gland network. Many pathways related to reproduction were identified, including TGFβ signaling ($P = 6.71 \times 10^{-5}$), Wnt signaling ($P = 4.1 \times 10^{-2}$), and peroxisome proliferator-activated receptor (**PPAR**) signaling ($P = 4.84 \times 10^{-2}$; corrected *P*-values; Supplementary Table S5 [see the online version of the article at http://journalofanimalscience.org]). The link between these pathways and reproduction is further deliberated in the discussion section.

Comparing the pituitary gland transcriptome between Brahman (current study) and Brangus heifers (Cánovas et al., 2014a), we found 11 genes that were DEx in both breeds ($P < 0.05$): *GPX6*, *DMBX1*, *IYD*, *C4BPA*, *MAG*, *LHX1*, *EXPI*, *LPO*, *SAA3*, *P2RY8*, and a novel gene (uncharacterized protein). Out of these 11, only 4 genes had the same expression pattern in both Brahman and Brangus, similarly up- or downregulated after puberty, and these were *GPX6*, *IYD*, *EXPI*, and *SAA3*. The other seven genes were reversed in their expression pattern: instead of being upregulated, they were downregulated in postpubertal heifers or vice versa (Supplementary Table S6; see the online version of the article at http://journalofanimalscience.org). These genes had a $|FC| > 1.87$ in both breeds, except only for *EXPI*, which had a FC = −1.27 in Brahman heifers and a FC =

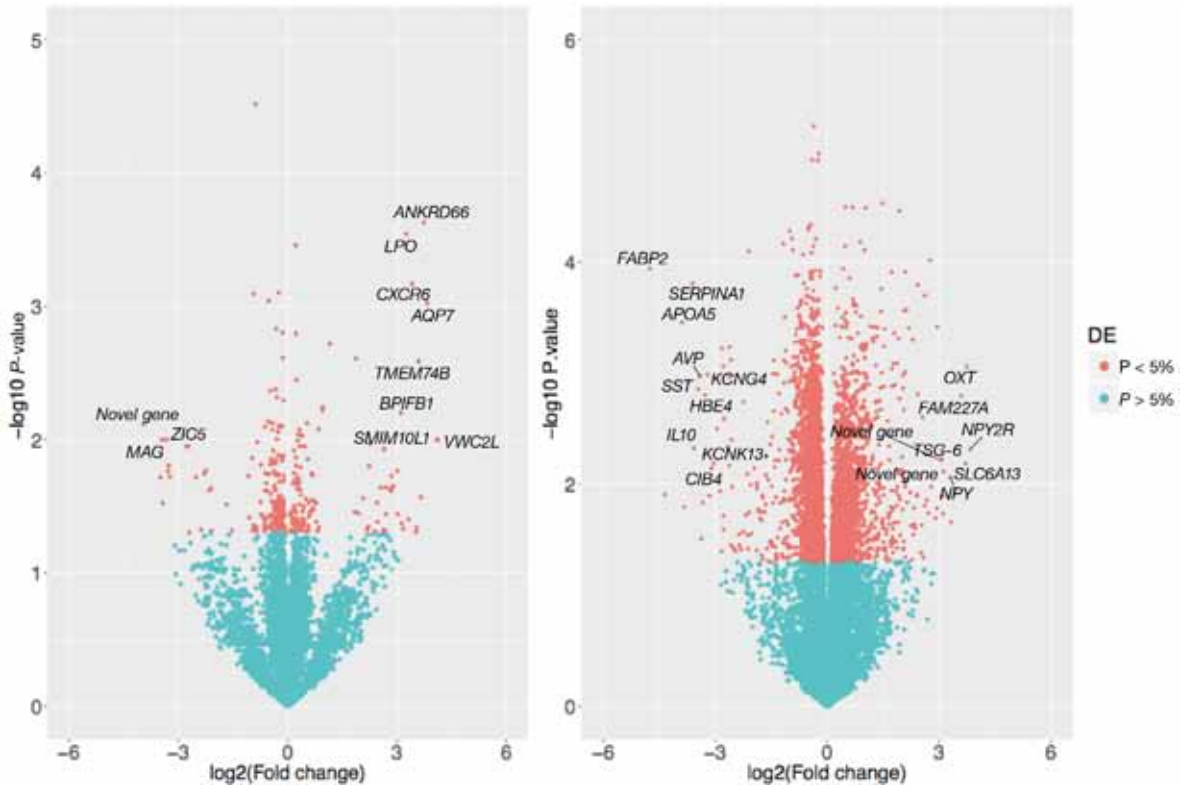

Figure 1. Volcano plots reveal genes that significantly differ between pre- vs. postpubertal heifers in the pituitary (left panel) and ovaries (right panel). The *x*-axis represents the fold change and the *y*-axis represents statistical significance for each gene. Red indicates genes that significantly differ ($P < 0.05$) between 2 groups. Genes symbols are provided for genes with a |fold change| ≥ 3 and $P \leq 0.01$.

−0.85 in Brangus heifers. Out of seven genes that were different with regards to the expression pattern in two breeds, five were downregulated in Brangus and upregulated in postpubertal Brahman heifers.

Ovarian Tissue Results

The number of DEx genes detected in ovarian tissues was 3,871 (Supplementary Table S7; see the online version of the article at http://journalofanimalscience.org). In ovaries, 2,434 genes were downregulated and 1,437 genes were upregulated after puberty. Just over 2% of these DEx genes had a |FC| ≥ 2 (either up or down). Genes highly DEx (|FC| ≥ 3, $P \leq 0.01$) in the ovary are shown in Fig. 1 and Table 2. For example, *SLC6A13* (a γ-aminobutyric acid [**GABA**] transporter), *OXT* (*oxytocin*), and *NPY* (*neuropeptide Y*) and its receptor *NPY2R* were among the DEx genes with higher FC. Their potential role in puberty is further explored in the discussion. Among ovarian DEx genes, 367 were TF, 119 were transcription cofactors, and 56 were chromatin remodeling factors. Further investigation of TF was executed using the RIF metrics.

Enrichment analyses of GO terms were performed using the 3,871 DEx genes as the target gene list and the genes expressed in both tissues (16,978 genes) as a background list. Ovarian DEx genes were enriched for 38 GO terms and 7 Kyoto Encyclopedia of Genes and Genomes pathways (Supplementary Table S8; see the online version of the article at http://journalofanimalscience.org). The GO terms included zinc ion binding and many terms associated with mitochondrial, metabolic, and catabolic activities, including the most significant GO term, "generation of precursor metabolites and energy" ($P < 5 \times 10^{-12}$). The pathways deemed enriched included "glycolysis/gluconeogenesis" and "steroid biosynthesis." Furthermore, seven zinc finger regions (defined as sequence features) were enriched for in the ovarian gene list.

Using the RIF metrics, we identified 96 significant top-ranking TF ($P < 0.05$) in the ovaries (Supplementary Table S9; see the online version of the article at http://journalofanimalscience.org). We identified 27 TF that belonged to the zinc finger family. In the ovary, the TF *ZNF84*, *NR3C2*, *ZNF354B*, *SOX8*, and *DMBX1* had the highest RIF1 scores (>3 SD units). All of these high-scoring TF were less expressed in postpubertal heifers ($P < 0.05$). No pathways were enriched in the TF ovarian gene list, but several GO terms related to transcriptional regulation showed enrichment, including the seven zinc finger regions (defined as sequence features) that were enriched for in the DEx ovarian gene list (Supplementary Table S10; see the online version of the article at http://journalofanimalscience.org).

Table 2. The reads per kilobase per million mapped reads (RPKM) values for genes that significantly differ in expression between pre- vs. postpubertal heifers in the pituitary and ovaries (|fold change| > 3, $P < 0.01$)

Tissue	ENSB tag[1]	Gene[2]	RPKM_PRE[3]	RPKM_POST[4]	FC[5]
Pituitary	ENSBTAG00000030199	Novel gene	4.538	0.600	−3.938
	ENSBTAG00000017044	MAG	5.920	2.478	−3.443
	ENSBTAG00000046271	ZIC5	6.903	3.585	−3.318
	ENSBTAG00000015724	BPIFB1	1.076	4.181	3.105
	ENSBTAG00000012780	LPO	3.205	6.466	3.261
	ENSBTAG00000015708	CXCR6	3.206	6.623	3.416
	ENSBTAG00000018418	TMEM74B	2.018	5.611	3.593
	ENSBTAG00000047009	ANKRD66	2.761	6.499	3.738
	ENSBTAG00000020105	AQP7	3.052	6.879	3.827
	ENSBTAG00000036258	SMIM10L1	2.914	7.008	4.094
	ENSBTAG00000018552	VWC2L	1.991	6.110	4.119
Ovary	ENSBTAG00000017045	FABP2	7.503	2.750	−4.753
	ENSBTAG00000019764	APOA5	7.334	3.446	−3.888
	ENSBTAG00000018843	SERPINA1	4.585	0.983	−3.602
	ENSBTAG00000006685	IL10	5.777	2.209	−3.568
	ENSBTAG00000017312	SST	6.364	2.908	−3.456
	ENSBTAG00000008027	AVP	6.432	3.027	−3.405
	ENSBTAG00000039178	HBE4	6.518	3.246	−3.272
	ENSBTAG00000016695	KCNG4	4.972	1.765	−3.207
	ENSBTAG00000005041	CIB4	4.560	1.458	−3.101
	ENSBTAG00000045849	KCNK13	5.105	2.064	−3.041
	ENSBTAG00000000198	LOC781146	0.817	3.824	3.008
	ENSBTAG00000007239	TSG-6	0.702	3.783	3.081
	ENSBTAG00000047490	Novel gene	0.990	4.112	3.122
	ENSBTAG00000004503	NPY	2.764	6.069	3.305
	ENSBTAG00000010573	FAM227A	1.650	5.253	3.603
	ENSBTAG00000014525	SLC6A13	3.087	6.799	3.712
	ENSBTAG00000008026	OXT	0.459	4.216	3.756
	ENSBTAG00000016633	NPY2R	1.288	5.100	3.812

[1]ENSB tag = Ensembl gene identifier according to http://uswest.ensembl.org/index.html (accessed 24 May 2016).

[2]Gene symbol related to the ENSB tag.

[3]RPKM_PRE = the RPKM in prepubertal heifers (average).

[4]RPKM_POST = the RPKM in postpubertal heifers (average).

[5]FC = fold change (RPKM _PRE minus RPKM_POST).

Coexpression networks were predicted with PCIT for the ovaries, using the expression values for DEx genes and all expressed TF in our samples (1,085 TF). The predicted network from ovarian results had 23,169 connections for 2,803 genes (Fig. 2B). Due to the large number of connections (23,169 connections) in the predicted ovarian network, a subnetwork comprising the significant coexpression correlations between top-ranking TF and highly DEx genes (|FC| > 1, $P \leq 0.01$) was explored (Fig. 2C). In the ovarian network, 29 genes were both top-ranking TF (RIF score > 1.96) and DEx genes ($P < 0.05$). Of these 29 genes, 20 genes belong to the zinc finger family. *ZNF84* and *ZNF354B* had the highest RIF1 score (>3 SD units) and these TF had 4 and 6 connections in the ovarian network. The gene *ZNF184*, with a RIF1 score of −2.54, had 105 connections in the network. In the ovarian subnetwork, zinc finger genes were noted as key regulators.

Pathway analysis showed enrichment for nine pathways in the ovarian network ($P < 0.05$). Within these nine enrichment pathways, the steroid biosynthesis pathway ($P = 3.42 \times 10^{-7}$) was observed. This is consistent with the fact that the major role of ovarian tissue is to produce steroid hormones with reproductive function. Other significant pathways are presented in Supplementary Table S11 (see the online version of the article at http://journalofanimalscience.org).

Our study also compared the ovarian transcriptome of Brahman and Brangus heifers (Cánovas et al., 2014a). Results had 40 genes in common that were DEx genes in both Brahman and Brangus heifers ($P < 0.05$; Supplementary Table S6 [see the online version of the article at http://journalofanimalscience.org]). These genes had |FC| > 1 in both breeds, except for *STMN1*, *RPL15*, and *TNMD* values in Brahman heifers, which were lower. For example, *STMN1* had FC = −0.55 in

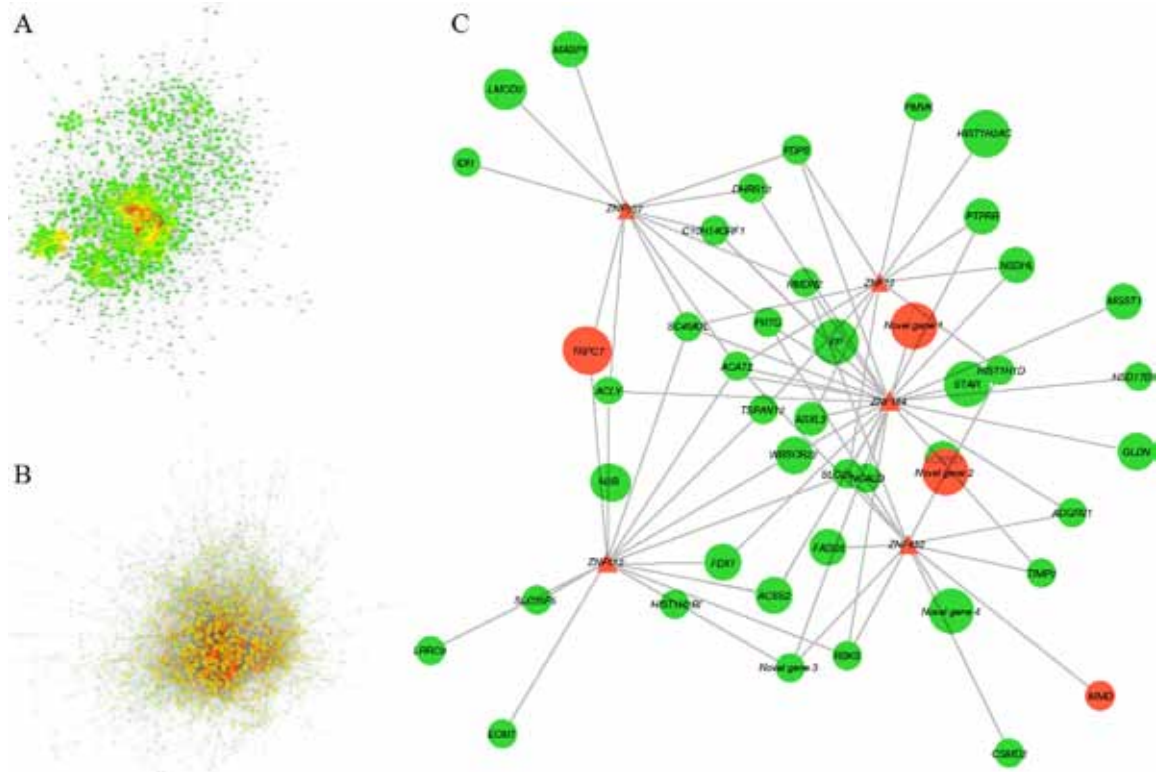

Figure 2. Predicted coexpression gene networks combining pre- and postpubertal data in the pituitary gland and ovarian tissue. Each node represents a gene or a transcription factor (TF). A) Pituitary coexpression network. B) Ovarian coexpression network. C) Subnetwork showing the key regulatory genes in ovaries. Nodes represented as triangles are TF and ellipses are all other genes. Edges represent significant predicted interaction between nodes. Node color indicates the number of connections of a specific gene in the network. The color spectrum ranges from green (low) to red (high) number of connections, except for panel C, where red nodes indicate downregulated genes and green nodes represent upregulated genes. Node size indicates the relative amount of expression in the postpuberty sample.

Brahman but FC = 4.09 in Brangus heifers. Moreover, out of 40 genes, 26 genes had a reversed expression pattern in these two breeds. For example, a reverse example was the expression of a GABA receptor (*GABRA5*) that was decreased in postpubertal Brangus heifers but increased in Brahman heifers. The expression of *OXT* was increased in postpubertal heifers in both breeds.

DISCUSSION

To our knowledge, only rwo other RNA sequencing studies have investigated heifer puberty. The first study used data from Brangus heifers, a cross between *B. indicus* and *B. taurus* (three-eighths Brahman and five-eighths Angus) cattle (Cánovas et al., 2014a). This Brangus heifer study discussed RNA sequencing results for multiple tissues, including the pituitary gland and ovaries (Cánovas et al., 2014a). The second study used the same Brahman animals reported herein to discuss gene expression changes in the hypothalamus of pre- versus postpubertal heifers (Fortes et al., 2016). Results reported herein for the pituitary gland and ovaries will be discussed in the context of these two previous studies.

The reference bovine genome sequenced is that of a *B. taurus* female (Bovine Genome Sequencing and Analysis Consortium et al., 2009). Bovine *B. taurus* and *B. indicus* subspecies diverged about 330,000 yr ago and so their genome sequences contain important differences (Bovine HapMap Consortium et al., 2009; Porto-Neto et al., 2013, 2014). Genomic differences and divergence can impact our results, as we have aligned a *B. indicus* transcriptome to the *B. taurus* reference genome to estimate gene expression. In the absence of a *B. indicus* reference genome, we currently must use the *B. taurus* reference and depend on high levels of gene homology. As soon as a *B. indicus* reference is available, resources such as the ones presented herein can help annotate that reference and serve for more refined comparisons between *B. indicus* and *B. taurus* gene expression, including annotation of specific isoforms.

The difference in content of *B. taurus* genetics, which is higher in Brangus than in Brahman, could explain the differences in our results, at least partially. The DEx genes in common between the two breeds were 11 in the pituitary gland and 40 in the ovaries. More than half of these DEx genes had reversed expression patterns (up- turned downregulated and vice versa) in Brahman heifers compared with Brangus heifers. Although the reversed expression pattern is puzzling, the differential expression pattern of these DEx genes

in two bovine breeds enhanced our confidence about their potential role in cattle puberty. Therefore, these reversed DEx genes could serve as a starting point to elucidate the differences in pubertal development between these two breeds and warrant further investigation.

Differences in the experimental design and number of studied animals should be taken into account when comparing the Cánovas et al. (2014a) study with our results. The prepubertal Brangus heifers were much younger (353 versus 437 ± 25 d of age) and smaller (290 versus 355 ± 30 kg) than the postpubertal heifers, which is in contrast to the present study, where pre- and postpubertal heifers were similar in terms of weight and age. No significant difference in BW or condition score between pre- and postpubertal heifers was observed at tissue harvest in our study (Fortes et al., 2016). Despite these differences, we still believe it is useful to compare both experiments.

Pituitary Gland Genes: Differentially Expressed and Interacting

The pituitary gland had 284 DEx genes in Brahman heifers compared with 292 DEx genes in Brangus heifers. Only 11 genes were DEx in both breeds ($P < 0.05$): *GPX6*, *DMBX1*, *IYD*, *C4BPA*, *MAG*, *LHX1*, *EXPI*, *LPO*, *SAA3*, *P2RY8*, and a novel gene (uncharacterized protein). Out of 11 genes, four had the same expression pattern in both Brahman and Brangus: *GPX6*, *IYD*, *EXPI*, and *SAA3*. In pigs, *GPX6* was proposed as a peripheral blood marker for early pregnancy detection (Shen et al., 2014). The early pregnancy scenario may be similar to postpubertal conditions in the luteal phase experienced by the Brahman and Brangus heifers. The gene *IYD* was also observed as a DEx gene in the hypothalamus of Brahman heifers (Fortes et al., 2016) as well as the pituitary gland of Brahman and Brangus heifers. Known to be involved in thyroid hormone biosynthesis, *IYD*'s role in reproductive tissues is less clear. The gene *EXPI* expressed in uterus of mice was related to pregnancy success (Nuno-Ayala et al., 2012). Its expression in the pituitary gland is reported here and in the Brangus study, but no further literature is available to illustrate *EXPI*'s potential role in this tissue. Genes of the serum amyloid A (SAA) family, such as *SAA3*, were expressed in the human pituitary gland (Urieli-Shoval et al., 1998), although its known role is in the immune system. In short, *GPX6*, *IYD*, *EXPI*, and *SAA3* were supported by both experiments and merit further investigation to clarify their roles in the pituitary gland of pubertal heifers.

The most DEx genes ($|FC| > 4$) in the pituitary gland of Brahman heifers were *VWC2L* (FC = 4.12, $P = 0.01$) and an uncharacterized protein (ENSBTAG00000036258 or *SMIM10L1*; FC = 4.09, $P = 0.01$). The gene *VWC2L* is known to be expressed in the brain, but its function is better understood in bones, where its expression was associated with osteoblast matrix mineralization (Ohyama et al., 2012). The effects of *VWC2L* might be through members of the TGFβ superfamily, such as the bone morphogenetic protein (**BMP**) it antagonizes (Miwa et al., 2009). In rats, *TGFβ1* induces the expression of GnRH (Srivastava et al., 2014). There is strong evidence for TGFβ superfamily signaling in the pituitary gland (Qian et al., 1996; Bilezikjian et al., 2006). In humans, an association between bone mineralization and pubertal development has been observed (Eastell, 2005). A functional role for *VWC2L* in cattle puberty, potentially via TGFβ signaling in the pituitary gland, is a new hypothesis emerging from our results. Further investigation into *VWC2L* may help elucidate the mechanisms that link bone mineralization and pubertal development in cattle and serve for comparisons with other mammals. The role that *SMIM10L1* (*small integral membrane protein 10 like 1*) may play in puberty is difficult to speculate, given the scarcity of data on this uncharacterized protein.

Pathway analysis using the list of pituitary gland DEx genes, key TF, and genes in the predicted network of the pituitary gland revealed enrichment for the TGFβ signaling pathway, Wnt signaling pathway, and PPAR signaling pathway.

The TGFβ superfamily signaling plays a pivotal role in the regulation of cell differentiation, growth, morphogenesis, tissue homeostasis, and regeneration (Massagué, 2012). *Transforming growth factor*-β (*TGF*β), a member of TGFβ superfamily, can increase GnRH gene expression as well as GnRH release (Prevot, 2002; Mahesh et al., 2006; Ojeda et al., 2010). Genes of this superfamily pathway detected in the pituitary gland network include *SMAD6*, *SMAD4*, *TFDP1*, *SP1*, *SMAD2*, *SMAD7*, *PITX2*, *SMAD3*, *E2F4*, *FOXJ1*, *SMAD1*, *ID1*, *ID3*, *ID2*, *MYC*, and *CHRD*. Among these detected genes, only *CHRD* was observed as downregulated after puberty (FC = −0.25, $P = 0.03$). *Chordin*, or *CHRD*, is an antagonist of BMP activity (Larrain et al., 2000; Oelgeschläger et al., 2000). Bone morphogenetic proteins play significant roles in follicular development (Juengel et al., 2004; Hussein et al., 2005) and a variety of physiological processes such as embryonic development, tissue homeostasis, apoptosis, proliferation, migration, differentiation, and bone formation (Urist, 1965; Wu and Hill, 2009; Wagner et al., 2010). More specifically to pituitary gland function, BMP receptors *SMAD4* and *SMAD3* were related to signaling for FSH transcription (Rejon et al., 2013; Fortin et al., 2014). Therefore, TGFβ superfamily genes could be involved in regulation of FSH secretion in pubertal Brahman heifers.

Genes in the pituitary gland network involved in the Wnt signaling pathway included *TP53*, SMAD

genes, *RACI*, and *NFAT5*, among others. *Tumor antigen p53* (*TP53*; RIF2 = 2.81) was associated with pituitary gland cell turnover, a process linked to the pituitary gland plasticity that is important for pubertal development (Garcia-Lavandeira et al., 2015). Note that SMAD genes belong to both the Wnt and the TGFβ signaling pathways. Interestingly, *RAC1* was reported to regulate several reproductive events, including embryo implantation (Grewal et al., 2008; Nicola et al., 2008), meiotic spindle stability and anchoring in mammalian oocytes (Halet and Carroll, 2007), and embryonic epithelial morphogenesis (He et al., 2010). In addition, *RAC1* can stimulate *STAT3* activation and therefore affect the transcription of many genes (Faruqi et al., 2001; Park et al., 2004; Kawashima et al., 2006). The deletion of *STAT3* reduced *POMC* levels and raised *AgRP* and *NPY* levels, resulting in obesity, hyperphagia, thermal dysregulation, and infertility (Gao et al., 2004). In pituitary gland cells, *RAC1* is thought to take part of the intracellular signaling that responds to GnRH secretion from the hypothalamus (Kraus et al., 2001). Perhaps this pituitary gland response to GnRH signaling explains the negative FC observed for *RAC1*, as progesterone levels in the postpubertal heifers create a context of negative feedback to GnRH production, with consequences in the pituitary gland. The TF *NFAT5* present in the pituitary gland network was also considered a key TF in the hypothalamus of Brahman heifers (Fortes et al., 2016). This TF was also identified in the Brangus study of heifer puberty (Cánovas et al., 2014a). Mutations in *NFAT5* were associated with puberty in women (Chen et al., 2012). These pituitary gland results and mentioned gene function warrant further investigation of *TP53*, SMAD genes, *RAC1*, and *NFAT5* in the context of cattle puberty.

Genes in the pituitary gland network that were annotated to the PPAR signaling pathway were *AQP7*, *CYP4A22*, *ILK*, *RXRA*, *RXRB*, *PPARG*, *NR1H3*, *PPARA*, *RXRG*, and *PPARD*. The TF *PPARG* was previously identified as a key regulator of cattle puberty, in a network prediction from genomewide association data (Fortes et al., 2010). It is feasible to speculate that PPAR signaling is important for puberty, because of its relevance to fat metabolism and the well-known interplay between nutrition, metabolism, and puberty in cattle (Cardoso et al., 2014, 2015).

Three among the PPAR signaling genes were DEx. Among these, *AQP7* had a high FC (3.83; $P = 0.0009$). The gene *AQP7* is a member of a small family that facilitates rapid passive movement of water (Huang et al., 2006). Water movement across cell membranes is important in processes underlying reproduction, and 11 isoforms of the aquaporin (**AQP**) family were reported as expressed in ovaries, testis, and embryos (Huang et al., 2006). Our study revealed upregulation of *AQP7* expression in the pituitary gland of postpubertal Brahman heifers. The expression of AQP genes can be mediated by steroid sex hormones (Jablonski et al., 2003; Richard et al., 2003; Lindsay and Murphy, 2004; He et al., 2006). High progesterone levels in postpubertal heifers contrasting with low levels in the prepubertal group may influence DEx results for *AQP7* in the pituitary gland. Further research is required to understand the specific role of *AQP7* in the bovine pituitary gland.

The gene *CYP4A22*, another PPAR signaling gene upregulated in postpubertal heifers, belongs to a cytochrome P450 family that is important for steroid synthesis (Pikuleva and Waterman, 2013). Pituitary gland expression of a cytochrome P450 family member suggests that this aromatase would be related to the mechanisms of action of gonadal steroids on pituitary gland differentiation and secretion, as shown by immunohistochemistry in rats (Carretero et al., 2003). The gene *CYP4A22* could be part of the pathways in the pituitary gland that respond to progesterone signaling, linking pituitary gland function to ovarian feedback mechanisms established with puberty.

The third DEx gene in the PPAR signaling pathway was *ILK*, known as an essential upstream regulator of Akt activation (Troussard et al., 2003). Notably, *KiSS-1* expression is mediated by an IGF1/Akt/mechanistic target of rapamycin (**mTOR**) pathway; therefore, activation of Akt is relevant for puberty (Hiney et al., 2010; Srivastava et al., 2016). The expression of *KiSS-1*, fundamental for GnRH release at puberty, was modulated by mTOR in prepubertal female rats, and mTOR may also be a link between leptin signaling and GnRH release (Roa et al., 2009). Evidence for the role of the *KiSS-1* pathway directly influencing pituitary gland activity is mounting (Gahete et al., 2016). The fact that *ILK* was DEx in the pituitary gland may serve as further evidence for this direct influence of the *KiSS-1* pathway on pituitary gland function (not only via hypothalamic signaling and GnRH release). Our results and the fact that *ILK* is essential for PPAR signaling and Akt regulation (which, in turn, is important for *KiSS-1* expression) raise the possibility that *ILK* is another molecular link between energy metabolism and the onset of puberty in cattle.

Ovarian Genes: Differentially Expressed and Interacting

In ovaries, 3,871 genes were DEx in our study, whereas only 311 genes were DEx in Brangus heifers. This large numerical difference in terms of DEx genes was probably influenced by differences in terms of experimental design. Although the Brangus study sequenced only eight ovaries (those from four

prepubertal heifers and four postpubertal heifers), the Brahman data we present is based on the sequencing of 24 ovaries (12 from the six prepubertal heifers and 12 from six postpubertal heifers). Sequencing both left and right ovaries separately probably increased our data set and the power for detecting DEx genes.

We detected 40 genes DEx in common between the Brahman and Brangus experiments. Both studies showed DEx results for *GABRA5* (a GABA receptor), although the expression pattern was reversed in postpubertal heifers: downregulated in Brangus and upregulated in Brahman. Specifically, 26 genes out of these 40 presented reversed expression patterns. Still, the suggestion that GABA signaling plays a role in ovarian function remains. In mice, GABA signaling was linked to oocyte maturation in ovaries (Dai et al., 2016).

Among the 3,871 DEx genes, many genes with higher expression in ovaries of postpubertal heifers in the luteal phase may play a role in puberty onset and in the feedback mechanisms to progesterone. Among DEx genes with a |FC| > 3 in the ovarian tissue, four were noteworthy for their known physiological functions: *OXT*, *SLC6A13*, and *NPY* and its receptor *NPY2R*.

The Cánovas et al. (2014a) study also noted the upregulated expression of the *OXT* gene in ovarian tissues of postpubertal heifers, in agreement with our findings. Our study reported higher expression of *OXT* after puberty, with a FC of 3.75 ($P = 0.0008$). The *OXT* gene is a posterior pituitary gland peptide hormone involved in many physiological events including lactation, cognition, adaptation, tolerance, and complex sexual and maternal behavior (Gimpl and Fahrenholz, 2001). In rats, oxytocin modulates the pulse and frequency of GnRH, facilitating pubertal development (Parent et al., 2008). In the ovary, oxytocin is known to contribute to luteolysis (Romero et al., 2013). It is possible that higher *OXT* expression in postpubertal heifers may indicate the next event in the estrus cycle following the luteal phase.

In our study using ovarian data, the FC of *SLC6A13* was 3.71, which increased after puberty ($P = 0.006$). The *SLC6A13* (also known as *GAT2*) is a member of solute carrier family 6 (neurotransmitter transporter, GABA), one of four GABA transporters. Notably, GABA transporters play a pivotal role in the regulation of the magnitude and duration of GABA's action (Hu et al., 2004). An increase of GABA transporters' activity at the onset of puberty might decrease GABA concentration in stalk-median eminence (Terasawa, 2005). Not only expressed in mammalian central nervous system, GABA and GABA receptors have also been demonstrated in relatively high concentration in the human ovary, rat ovary, and rat fallopian tube as well as other mammalian reproductive tissues (Martin del Rio and Caballero, 1980; Erdö and László, 1984; Akinci and Schofield, 1999; Biggs et al., 2013; Martyniuk et al., 2013). Our study was the first to reveal the expression of *GAT2* in *B. indicus* ovaries. Furthermore, *GAT2*/*SLC6A13* was observed at the blood brain barrier and it contributes to GABA transport (Takanaga et al., 2001; Zhou et al., 2012). Although there is no previous information on *SLC6A13* activity in ovaries (or cattle puberty in general), the increase of *SLC6A13* after puberty in Brahman heifers and the relation between *SLC6A13* and GABA concentration should be noted, as *SLC6A13* is potentially a puberty-activating gene in heifers.

Two significant DEx genes in ovaries, *neuropeptide Y* (*NPY*) and *NPY2R* (*neuropeptide Y receptor Y2*) were observed in our study, with FC of 3.81 ($P = 0.004$) and 3.30 ($P = 0.008$), respectively. Notably, *NPY* regulates the secretion of GnRH from the hypothalamus and also affects anterior pituitary gland hormone release (McDonald et al., 1989; Terasawa, 1995). Indeed, *NPY* regulates the secretion of GnRH and LH in rodents (Kalra, 1993), sheep (Morrison et al., 2003), pigs (Barb et al., 2006), and cattle (Thomas et al., 1999). The regulatory role of *NPY* in GnRH secretion is complex and dependent on the steroid environment, as reviewed by Celik et al. (2015). In particular, when a high concentration of estrogen level is present, *NPY* stimulates the secretion of GnRH, and when estrogen levels are low, *NPY* inhibits GnRH release (Celik et al., 2015). Although *NPY* is mostly expressed in the mammalian brain, it is also expressed in human primary ovarian neoplasms (Körner et al., 2003), in rat ovaries (McDonald et al., 1987), and in bovine ovaries (Hulshof et al., 1994) as well as ovine ovaries (Keator et al., 2010). Our study showed differential expression of *NPY* and *NPY2R* in ovaries when pre- and postpubertal heifers were compared. As *NPY* can act directly on the secretion of progesterone and oxytocin from bovine CL (Miyamoto et al., 1993), its role in postpubertal heifer on diestrus can be speculated. The presence of *NPY* at the hypothalamic–pituitary–gonada axis and its effect on the secretion of GnRH and LH as well as progesterone and *OXT* indicate its complex effect on reproductive physiology at several levels (McDonald et al., 1987). Further investigation of *NPY* and *NPY2R* could help to elucidate the role of these genes and associated pathway in ovarian tissue related to puberty onset.

Pathway analysis using the list of genes in the predicted ovarian gene network as target revealed enrichment for nine pathways (Supplementary Table S11; see the online version of the article at http://journalofanimalscience.org). Among these pathways, the steroid biosynthesis pathway was significant ($P = 1.49 \times 10^{-5}$), probably as a result of steroidogenesis being a major ovarian function and the fact that the pre- versus postpubertal heifers comparison investigated two very different ovarian scenarios: the postpubertal animals were producing

progesterone. All genes annotated to the steroid biosynthesis pathway were DEx genes upregulated after puberty; they were *NSDHL*, *DHCR24*, *DHCR7*, *LSS*, *LIPA*, *CYP51A1*, *HSD17B7*, *EBP*, *SC4MOL*, *SC5DL*, *FDFT1*, *SQLE*, and *TM7SF2*. The FC for these genes ranged from 0.52 to 1.89. The most upregulated gene in this pathway was *TM7SF2* (FC = 1.89, $P = 0.003$) followed by *DHCR24* (FC = 1.44, $P = 0.002$), *DHCR7* (FC = 1.31, $P = 0.001$), and *NSDHL* (FC = 1.3, $P = 0.001$). These DEx genes are involved in cholesterol conversion into steroids, such as progesterone (Hanukoglu, 1992; Rone et al., 2009; Miller and Bose, 2011).

From the large predicted network on the ovary, we explored a subnetwork with selected genes based on connectivity degree (numbers of connections), top-ranking TF (significant in RIF), and DEx genes ($|FC| \geq 1$, $P \leq 0.01$). We identified *ZNF507*, *ZNF12*, *ZNF512*, *ZNF184*, and *ZNF432* as hubs of this subnetwork. Note that in the predicted ovarian network, the *ZNF432* seems to be a key TF with 131 targets, which was followed by 105 targets predicted for *ZNF184* and 87 targets of *ZNF512*. These five TF were all zinc fingers that were downregulated after puberty. This result confirmed previous studies noting the decrease of zinc finger gene expression after puberty in monkeys (Lomniczi et al., 2015).

Our overall ovarian results confirmed the decrease in expression of zinc finger gene expression in postpubertal Brahman heifers: 19 key zinc fingers were downregulated ($FC \leq -0.3$, $P < 0.05$). Only *ZNF891* expression was increased in postpubertal Brahman heifers (FC = 0.76, $P = 0.048$). The role of these DEx zinc fingers in bovine ovaries needs further investigation. Four novel genes were predicted zinc finger targets in the subnetwork, 2 upregulated and 2 downregulated; these results are the first report of their potential function as puberty-related genes.

Zinc Fingers Expressed in the Hypothalamus, Pituitary Gland, and Ovaries

Lomniczi et al. (2015) reported zinc finger genes as a subnetwork controlling the onset of female puberty. In the studied Brahman heifers, zinc fingers were DEx and/or important TF in the hypothalamus (Fortes et al., 2016), the pituitary gland, and the ovaries (as reported herein). We consider the possibility of zinc finger expression being ubiquitous in bovine tissues. However, each of the investigated tissues had its specific zinc fingers that were DEx and/or key TF (not all were the same across tissues). Most DEx genes were tissue specific: the hypothalamus and the pituitary gland had 8 DEx genes in common, and the hypothalamus and ovaries had 89 DEx genes in common. Zinc fingers emerged as important TF across all 3 tissues of the reproductive axis. According to the RIF metrics, 26 (hypothalamus), 22 (pituitary gland), and 28% (ovaries) of TF identified as important regulators of puberty in Brahman heifers coded for genes of the zinc finger family.

Among key zinc finger TF, 2 were the same for the pituitary gland and ovaries: *ZNF219* and *ZSCAN26*. These were 2 out of only 7 TF that had significant RIF for both tissues. In the hypothalamus (Fortes et al., 2016) and pituitary gland (current results), the genes *ZNF791* and *ZNF576* were deemed important zinc finger TF in Brahman heifers. The key zinc finger TF in common between ovaries and the hypothalamus were *ZFHX2* and *ZNF576*. Although *ZNF576* had a significant RIF score in all 3 tissues of the hypothalamus–pituitary–gonadal axis, the majority of key zinc finger TF were significant in a tissue-specific manner. Although some zinc fingers may be ubiquitously expressed, we have identified zinc fingers that are relevant for only 1, 2, or 3 tissues in the reproductive axis. Future endeavors to understand their various roles will need to consider specific tissue context.

The potential role of genes belonging to the zinc finger family in the pubertal process was proposed by previous genome-wide association studies: associations between single nucleotide polymorphisms located near *ZNF462* and *ZNF483* and age of menarche were identified in women (Perry et al., 2009; Elks et al., 2010; Chen et al., 2012; Demerath et al., 2013). Furthermore, studies in male and female mice indicated that there was an increase of *MKRN3*/*ZNF127* mRNA levels in prepubertal heifers, a reduction in levels immediately before puberty, and low levels after puberty (Abreu et al., 2013). Decreased expression of zinc finger genes including *GATAD1* and *ZNF573* in peripubertal female monkeys was reported (Lomniczi et al., 2015). In fact, *GATAD1* was suggested to have a dual role in both contributing to GnRH release at the occurrence of puberty and silencing GnRH pulse generator at the infantile–early juvenile transition. This study also noted that the *GATAD1* gene can directly repress the transcription of 2 puberty-activating genes, *KiSS-1* and *TAC3*. The downstream effect of *KiSS-1* and *TAC3* can subsequently influence hypothalamic release of GnRH. Zinc fingers pathways to impact on pituitary gland and ovarian function are less clear.

Summary and Conclusions

Transcriptome results from reproductive tissues from developing Brahman and Brangus heifers highlight the importance of TF zinc fingers. The 2 zinc finger genes considered key TF in the hypothalamus were downregulated after puberty (Fortes et al., 2016), similar to the discussed ovarian results. Interactions between zinc finger genes and predicted target genes

need validation beyond our coexpression results. Interactions between zinc finger genes and targets considered puberty-activating genes are of particular interest to further unveil the proposed mechanisms of puberty (Lomniczi et al., 2015). Before puberty, zinc finger genes are proposed to inhibit puberty-activating genes. When the expression of zinc fingers decreased after puberty, it could result in the increase of puberty-activating genes, contributing to puberty onset.

In summary, reported RNA sequencing data from pre- versus postpubertal Brahman heifers in the pituitary gland and ovaries were used to identify key TF regulators of DEx genes and to predict coexpression networks. Reported results corroborate some of the known biology of puberty, recapitulating genes and pathways involved in GnRH secretion, steroidogenesis, and ovarian feedback mechanisms to the hypothalamus and pituitary gland. Tissue-specific zinc finger TF seemed particularly important regulators of gene expression in the hypothalamus–pituitary–gonadal axis of pre- versus postpubertal Brahman heifers. Furthermore, novel genes were implicated in pubertal development, which require additional research for their characterization.

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