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Identification of predicted genes expressed differentially in pituitary gland tissue of young growing bulls revealed by cDNA-AFLP technique

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ABSTRACT: Differentially expressed transcript derived fragments (TDFs) of bovine pituitary gland tissue at different developmental ages of Limousine and Hereford bulls were identified by cDNA-AFLP technique. Study revealed comparatively higher differentially expressed transcripts in 6-month Limousine bulls and 12-month Hereford bulls. The BLASTn/p analysis identified 3 and 21 predicted genes which gave significant e-values for Limousine and Hereford respectively, in assembled *Bos taurus* genome. The identified predicted genes expressed in bovine pituitary gland showed their mapped positions on bovine chromosomes: BTA2, 3, 5, 8, 9, 11, 12, 15–23, 26, and 28, respectively. Results based on TDF annotation identified 10 sequences that have BLAST hits to known annotated bovine genes and 14 sequences to unannotated contig regions in the latest gene Ensembl database Btau_4.0. Two breed specific predicted target genes were validated by qRT-PCR. Within and between breeds, qRT-PCR results revealed highly significant differences in the expression levels of bovine euchromatic histone-lysine *N*-methyltransferase 1 (*EHMT1*) and NCK adaptor protein 2 (*NCK2*) predicted genes. Obtained results conclude that cDNA-AFLP is a reliable technique for studying within breed age dependent gene expression patterns.

Keywords: *Bos taurus*; breeds; differential display; gene expression; Hereford; Limousine; polyacrylamide gel electrophoresis; restriction enzymes; developmental ages; real time PCR

Genome research on postnatal growth and developmental processes in farm animals has long been of interest to animal scientists, since such research can lead to improvement of meat quality and productivity. As a compound endocrine organ, the mammalian pituitary gland plays a major role in postnatal growth and development through regulation of reproductive development and fitness, growth, metabolic homeostasis, lactation, and the response to stress, by actions on target organs such as the gonads, the liver, the thyroid, the adrenals, and the mammary gland (Savage et al., 2003). In cattle, it has direct relation with beef production, particularly through its influential hormonal and cellular role on bovine postnatal body/muscle growth and composition

traits. The pituitary's endocrine growth control involves complex interactions of several hormones and growth factors, acting in both an endocrine and a paracrine or autocrine manner. In cattle, postnatal body/muscle growth and development have been extensively studied (Reecy et al., 2003, 2006; Chester-Jones and Vellaman, 2008; Chung and Johnson, 2008). In past research, QTL studies have identified several QTL regions and candidate genes for postnatal growth and development traits (Polineni et al., 2006), bovine growth traits were identified on bovine chromosomes (BTA) 2, 6, 14, 19, 21, and 23 (Kneeland et al., 2004). Interestingly, QTL for postnatal muscularity at BTA2 and fatness at BTA21 were mapped to different regions of the genome (Afolayan, 2003). Based on the available bovine QTL database (http://www.animalgenome. org/cgibin/QTLdb/BT/index), QTL for postnatal growth and development were mapped in many regions of bovine chromosomes. These QTL regions are generally large and contain several putative causal genes (Hu et al., 2007). Traditionally, breeding and selection programs for postnatal growth and development are practiced and based on the conventional measurements of postnatal weight and changes in weight over time for the determination of growth across ages. It is expected that the component traits like body dimensions (e.g. height), muscularity (defined as ratio of stifle over hip width), and fat depth measurements with weight predict beef carcasses for yield and possibly for quality. Therefore, they are utilized for body composition evaluation in the global beef industry.

The screening for tissue specific differentially expressed genes is one of the most straightforward approaches to reveal the molecular basis of a biological system. As a differential screening method, cDNA-amplified fragment length polymorphism (cDNA-AFLP) is more stringent and reproducible than many other methods, because it can amplify low-abundance transcripts (Bachem et al., 1996). The cDNA-AFLP technique is a robust, high throughput, genome-wide expression tool for gene discovery, where prior knowledge of sequences is not required (Bachem et al., 1998). The cDNA-AFLP is widely utilized in plant genome research as a PCR-based expression profiling technique to identify differentially expressed genes (Wang et al., 2009, 2010; Henriquez and Daayf, 2010). However, fewer studies have been reported for the domestic animals (in dogs – Vandeput et al. (2005), in horses – Cappelli et al. (2005, 2007)).

A study in rats suggested that tissue and age specific mRNA expression patterns could explain novel developmental role in mammals (Stone and Nikolics, 1995). Here, we report the first experimental investigation of breed-, tissue-, and age-specific gene expression patterns of cDNA-AFLP in context of genetics improvement of bovine growth and development traits.

The aim of the present study is to perform the transcriptome profiling of pituitary gland tissue using cDNA-AFLP technique to identify differentially expressed patterns of novel transcripts (transcript derived fragments: TDFs) as well as to search for the predicted genes (expressed sequence tags: ESTs) involved in bovine postnatal growth and developmental processes. For this purpose, young growing bulls of Limousine and Hereford breeds at the age of 6, 9, and 12 months were studied. For each beef breed, differentially expressed patterns in pituitary gland tissue were observed at three developmental ages in young growing bulls followed by identification of predicted genes. Finally, the identified predicted genes were validated by qRT-PCR.

MATERIAL AND METHODS

To elucidate the molecular and biochemical mechanisms involved in bovine postnatal growth and developmental processes, we carried out the age and breed specific cDNA-AFLP analysis on RNA samples isolated from pituitary gland tissue of young growing bulls aged 6, 9, and 12 months from two selected cattle breeds. The young growing bulls at different developmental stages were chosen because the postnatal growth and development of cattle is well established during this time, and the overall assessment of functional role of pituitary gland on major component traits (average body weight and dimensions, muscular development, fatness, pre-weaning growth, carcass values, etc.) of bovine postnatal body growth and development can be achieved.

Animals and tissue collection

Experimental design was composed of young bulls from two beef breeds: Limousine ($n = 25$) and Hereford ($n = 25$). Pituitary gland tissue samples from young growing bulls aged 6 ($n = 5$),

Table 1. Average phenotypic values of body weight measured at different developmental ages in young Limousine and Hereford bulls

Breeds	Developmental ages (months)	n	Mean ± SEM
Limousine	6	5	117.17 ± 17.63
	9	5	287.80 ± 03.26
	12	15	366.25 ± 13.80
Hereford	6	5	173.00 ± 05.75
	9	5	223.80 ± 20.00
	12	15	352.09 ± 11.89

9 (n = 5), and 12 (n = 15) months were collected after slaughtering. Samples were transferred immediately to the liquid nitrogen and finally stored in deep freezer at −80°C. The experimental animals were kept and reared in the same herd under uniformed feeding and environmental conditions. The phenotypic parameter average body weights measured at different developmental ages in young growing bulls of Limousine and Hereford breeds are presented in Table 1. All procedures involving animals were performed in accordance with the guiding principles for the care and use of research animals, and were approved by the local ethics commission (permission No. 3/2005).

RNA extraction and cDNA synthesis

Total RNA was extracted and prepared from 50–60 mg of frozen pituitary gland (representing both anterior and posterior) tissues using the guanidinium thiocyanate (TRIzol reagent: Invitrogen, Carlsbad, USA) method (Chomczynski and Sacchi, 1987). To check the yield/quality of the isolated total RNA from pituitary gland tissues, the samples were analyzed both quantitatively by nanodrop spectrophotometer (Thermo Scientific, West Palm Beach, USA) and qualitatively by 2% denaturing agarose gel. The single-strand (ss) cDNA synthesis was performed using SuperScript™ III RT and oligo $(dT)_{20}$ primers (Invitrogen, Carlsbad, USA). For the qRT-PCR experiment, ss cDNA were synthesized from all samples for each breed (5 samples, each of 6 months and 9 months, and 15 samples of 12 months). However, a total of six ss cDNA pools were constructed by taking the equal aliquots of ss cDNA within the age group of 6 (n = 5), 9 (n = 5), and 12 (n = 15) months, representing beef breeds. The double-strand (ds) cDNA synthesis from six pooled samples was performed using

commercially available ds cDNA synthesis kit (Invitrogen, Carlsbad, USA). Finally, the synthesized ds cDNA templates were purified using commercially available DNA purification Wizard kit (Promega, Madison, USA) (Pareek, 2006).

cDNA-AFLP analysis

The cDNA-AFLP analysis was performed as described by Bachem's protocol (Bachem et al., 1998) but with the commercially available AFLP expression analysis kit of LI-COR (LI-COR Biosciences, Lincoln, USA). 100 ng of the pool of ds cDNA was used as the initial template and the manufacturer's instructions were followed to generate $TaqI^{+0}/MseI^{+0}$ pre-amplification PCR products, which were assayed for quality and quantity by electrophoresis on 1% agarose gels. The primer sequences of the $TaqI$ and $MseI$ were 5'-GTAGACTGCGTACCGAN-3' and 5'-GATGAGTCCTGAGTAAN-3, respectively. The pre-amplification products were diluted 1 : 300 in sterile water (Fermentas, Vilnius, Lithuania) and used as template for final selective amplification. Selective PCRs were performed by $TaqI^{+2}/MseI^{+2}$ primer combinations using eight $TaqI^{+2}$ primers (+GA, +GT, +TC, +TG, +CT, +CA, +AG, and +AC) and eight $MseI^{+2}$ primers (+AC, +AG, +CA, +CT, +GA, +GT, +TC, and +TG) provided in the AFLP expression analysis kit. The $TaqI^{+2}$ primers in this kit are fluorescently labelled with infrared dye IRDye 800 (LI-COR Biosciences, Lincoln, USA) for the purpose of fragment visualization. 58 and 64 $TaqI^{+2}/MseI^{+2}$ were utilized to generate the pituitary gland tissue specific cDNA-AFLP gene expression profile patterns in young growing bulls of Hereford and Limousine breeds, respectively. Selective PCR products were resolved on 6.5% denaturing polyacrylamide gels (KBPlus Gel Matrix;

LI-COR Biosciences, Lincoln, USA) in DNA genetic analyzer (LI-COR Model 4200S). The cDNA-AFLP images were saved in 16-bit TIFF format for image analysis and the average number of TDFs generated per kb of cDNA sequence after final amplification with all 122 $TaqI^{+2}/MseI^{+2}$ primer combinations were calculated. In all generated transcriptomic profiles of bovine pituitary gland tissue, the expression patterns were identified as differentially displayed (DD), identically displayed (iDD), and single displayed (sDD) on the basis of presence/absence of TDF bands in each age group (Figure 1). In transcriptome profile, the sDD and DD mean the presence of TDF bands only in one and two age groups, respectively. However, iDD means the presence of TDF bands in all age groups.

TDFs band extraction

Band excision of targeted differentially expressed TDFs bands was performed using LI-COR Global Edition IR2 DNA Gene Sequencer Analyzer and the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, USA). Following partial 6.5% PAGE on LI-COR DNA analyzers, polyacrylamide gels containing fragments of interest were scan marked with the LI-COR Odyssey Infrared Imaging System. Bands corresponding to differentially expressed transcripts were cut from the gel with a sharp razor blade, with maximum care to avoid any contaminating fragment(s), resolved and eluted in TE buffer. Elution was achieved by five cycles of freezing (–20°C) and thawing. Eluted PCR products were reamplified with the same primer combination as in the corresponding final amplification reaction. The reamplification was done using the pre-amp primer mix as before, followed by a more selective re-amplification using LI-COR IRD800 labeled primers from the original reaction. Following re-amplification, the PCR products were resolved on a LI-COR gel alongside the original cDNA-AFLP fragments to confirm re-amplification of the correct TDF.

Direct sequencing of target TDFs bands

Before direct sequencing, the selective amplified PCR products were purified using purification column kit (Sigma, St. Louis, USA). The purity of DNA was checked quantitatively through nanodrop

spectrophotometer. The direct sequencing of DNA fragments were performed with MegaBACE 1000 sequencer (GE Healthcare Bio-Sciences Corp., Pittsburgh, USA) using routine standard protocol.

Data mining and annotation of putative TDFs

By using the BLAST-n (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) sequence alignment program, nucleotide sequences were compared with those in the NCBI non-redundant and *Bos taurus* databases (Altschul et al., 1997). Each best hit sequence was compared against newly assembled cow genome (Btau_4.0) database using the BLAST-n program (http://www.Ensembl.org/Bos_taurus/blastview). Significant differentially expressed predicted transcripts were identified based on the e-value (Altschul et al., 1997). Functional annotations were assigned to cluster sequences using online bioinformatics tools (see gene ontology (http://www.geneontology.org) (Khan et al., 2003; Hughes et al., 2008) and Blast2GO (http://blast2go.bioinfo.cipf.es/home) (Gotz et al., 2008)). The predicted gene/protein function in the NCBI database was searched against InterPro using the available online bioinformatics tool (http://www.uniprot.org) and determined according to Tao et al. (2007) and Chitale et al. (2009).

Quantitative gene expression analyses

Differentially expressed bovine TDFs sequences in context to bovine postnatal growth and developmental processes were identified by combined cDNA-AFLP and qRT-PCR analyses. The qRT-PCR was carried out on individually synthesized ss cDNA of the two breeds. The ss cDNA was synthesized as described earlier in this section using SuperScript™ III RT and oligo $(dT)_{20}$ primers (Invitrogen, Carlsbad, USA). All ss cDNA samples for each breed (n = 25) were examined in three technical replicates. To validate the cDNA-AFLP experiment, two TDF sequences of euchromatic histone-lysine N-methyltransferase 1 (*EHMT1*: Limousine) and NCK adaptor protein 2 (*NCK2*: Hereford), showing differential expression at different developmental age groups were selected for further quantitative gene expression analyses.

For real-time PCR assay, primer sequences of bovine reference gene *GAPDH* (NCBI reference sequence: NM_001034034.1) and *EHMT1* and

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Table 2. Primer sequences, annealing temperatures (T_a), and PCR product sizes of predicted target genes and bovine reference genes utilized in real time qRT-PCR study in bulls

Breed	Location	Reference and predicted target genes	Forward and reverse primers	Size (bp)	T_a (°C)
Bos taurus	BTA5	*GAPDH*	CATCATCTCTGCACCTTCTG AGGCATTGCTGACAATCTTG	95	51.8
Limousine	BTA11	*EHMT1*	CGCCAACATCGACACCTG CCCGGCCTTGATGAGGTA	100	52.6
Hereford	BTA11	*NCK2*	GCCTGGTCCCCTCTTTGT GCGGTGTGAATGGACTAAGG	158	53.2

NCK2 target genes were designed using Primer3 (Version 0.4.0) (Rozen and Skaletsky, 2000). The primers sequences, annealing temperatures, and PCR product sizes of selected reference and target genes utilized in this study to examine the levels of gene expression are provided in Table 2. The bovine *GADPH* gene was selected as a reference gene to normalize qRT-PCR experiments since it was validated among the most stably expressed genes tested in various bovine tissues, including pituitary gland tissues (Lisowski et al., 2008).

The qRT-PCR reaction mix (20 µl) consisted of 1.0 U of Dynazyme DNA polymerase (Finnzyme, Helsinki, Finland) with the 10x buffer containing 2.5mM $MgCl_2$, 0.25mM dNTP mix, 0.3µM of each primer, 0.75 µl of a 1/1000 stock dilution of Sybr-Green (Invitrogen, Carlsbad, USA), 0.6 µl of ss cDNA and sterile distilled water to a total volume of 20 µl. Reactions ran for 40 cycles at 95°C (20 s), 60°C (15 s), and 72°C (20 s) after initial denaturing at 95°C for 2 min. To ensure that each amplicon was a single product and detect possible primer dimerisation or other artifacts of amplification, a melting-curve analysis was performed immediately after completion of the real-time PCR (95°C 15 s, 60°C 15 s, and then slowly increasing the temperature to 95°C at a 2% ramp rate, with continuous measurement of fluorescence). Negative control reactions were included in each run and contained all reaction components except the template. All reactions were performed in triplicate, including three non-templates as the negative controls. Fluorescence was measured at the end of elongation stage of every PCR cycle.

Quantification of gene expression was performed using a Real-Time PCR system (Eppendorf AG, Hamburg, Germany). Threshold values (CT) generated from the Eppendorf software tool (Eppendorf AG, Hamburg, Germany) were employed to quantify relative gene expression. The relative expressions were corrected for the differences in the PCR efficiency. Subsequently the levels of each individual transcript were compared to the mean value of the group. The normalization was performed using suitable and unregulated references or housekeeping gene *GAPDH* (NCBI reference sequence: NM_001034034.1).

Statistical differences (analysis of variance: ANOVA) among the mRNA levels of predicted target genes expressed in the bovine pituitary gland were calculated by the General Linear Models (GLM) Procedure of SAS (Statistical Analysis System, Version 9.22, 2010).

$$Y_{ijkl} = \mu + A_i + B_j + T_k(AB)_{ij} + e_{ijkl}$$

where:

Y_{ijkl}	= value of expression level measured on each of the $ijkl$[th] animal
μ	= mean value common to all records
A_i	= within breed fixed effect of the age on predicted target genes (*EHMT1*: Limousine and *NCK2*: Hereford)
B_j	= fixed effect of the breed on predicted target genes
$T_k(AB)_{ij}$	= effect of the real-time PCR technical repetitions of individuals nested within age breed group
e_{ijkl}	= random error

RESULTS

Detection of expression patterns (iDD, DD, and sDD TDFs in each age group) at different developmental ages in growing bulls

The present study identified large number of anonymous differentially expressed DD, iDD, and sDD TDF bands using 122 PCR reactions of *Taq*I^{+2}/*Mse*I^{+2} primer combination in beef breeds
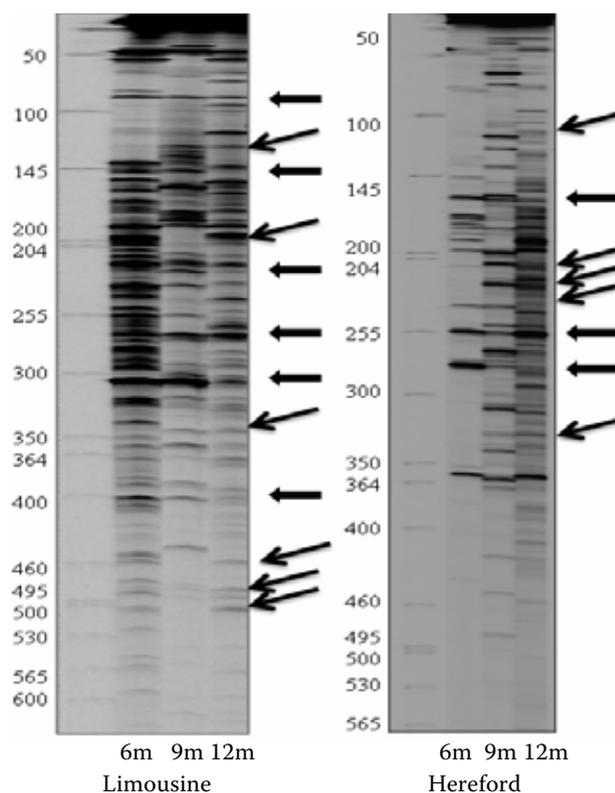
Figure 1. Expression patterns of within breed age specific transcripts revealed by cDNA-AFLP

An example showing selective amplification of primer combinations of *T-GA_M-AC* in Limousine and Hereford breeds. In each of within breed age specific cDNA-AFLP profiles, the extreme left are placed standard ladder, the straight arrow indicates iDD TDFs, and angled arrow indicates DD TDFs

AFLP = amplified fragment length polymorphism, iDD = identically displayed, DD = differentially displayed, TDFs = transcript derived fragments

(Table 3). On the average, 44 TDFs were visualized as bands and their lengths were 50–700 bp, depending upon primer combinations. Figure 1 shows an example of the expression patterns of the transcripts revealed in beef breeds using cDNA-AFLP with the primer pair of *T-GA_M-AC*. The cDNA-AFLP experiment revealed comparatively higher differentially expressed transcripts (TDFs) in Limousine than Hereford. However, within

breeds, higher and lower numbers of differentially expressed transcripts were observed in young bulls aged 6 and 12 months for Hereford and young bulls aged 12 and 6 months for Limousine (Table 3).

TDF band excision, direct sequencing, data mining of putative TDFs and sequence analysis

On the basis of highly differential display expression pattern and easiness to cut the bands from thin polyacrylamide gel, 20 and 40 DD TDFs bands were selected and the respective TDF bands were excised for Limousine and Hereford. A total of 16 and 32 TDF bands for Limousine and Hereford breeds were successfully recovered from gels and qualified for direct sequencing. The BLAST-n and BLAST-x of these 48 sequences against *Bos taurus* genomic sequence (http://www.Ensembl.org/Bos_taurus/blastview) identified 24 predicted gene transcripts expressed in bovine pituitary gland tissue, on the basis of having hits with non-significant e-values, i.e. > 0.01 (an alignment is considered statistically significant only when its e-value is smaller than 0.01) (Altschul et al., 1997). The BLAST analysis results excluded 24 transcripts which gave non-significant e-values. Breed-specific 3 and 21 predicted transcripts expressed in bovine pituitary gland were finally identified in Limousine and Hereford breeds, respectively (Table 4). In Limousine breed, identified predicted genes were the cytokine that regulates the *IL15*, *SNORA61*, and *EHMT1* genes. For Hereford breed, the identified predicted genes were *ID3*, *C1QA*, *MAN1A2*, *A5PKH9*, *SRP*, *SSH2*, *IRX1*, *CA201*, *COL24A1*, *GKAP1*, *JMJD2*, *KDM4C*, *NCK2*, *Q95M07*, *A1L539*, *TAGL*, *DDN1*, *SELK*, *SRGAP2*, *NPAS1*, and *5NTC*.

TDF annotation

Analysis of BLAST-n matches with significant e-values of 24 transcript sequences (Table 4) re-

Table 3. Summary of observed iDD, DD, and sDD TDFs bands in within breed age specific cDNA-AFLP experiment

Breeds	Primer pairs *Taq*I[+2]/*Mse*I[+2]	iDD	DD	sDD 6 months	sDD 9 months	sDD 12 months
Limousine	64	677	1100	1337	834	600
Hereford	58	282	844	430	548	1319

iDD = identically displayed, DD = differentially displayed, sDD = single displayed, TDFs = transcript derived fragments, AFLP = amplified fragment length polymorphism

vealed that 10 sequences have BLAST hits to known annotated bovine genes and 14 sequences have BLAST hits to unannotated contig regions in latest gene Ensembl database Btau_4.0 (http://www.Ensembl.org/Bos_taurus/Info/Index). The functional categories of BLAST hit to known bovine genes and unannotated contig regions were classified on the basis of their homology to known proteins (http://www.uniprot.org, http://www.geneontology.org). Each transcript was functionally annotated through careful analysis of the scientific literature and the gene ontology database (Khan et al., 2003; Hughes et al., 2008). It identified the following functional categories, namely DNA/RNA and metal binding protein, phosphoproteins, phosphorylation, signalling pathways, signal transduction, neurogenesis and ectoderm development, epidermal growth factor receptor. In general, the functional annotation allows identification of biological pathways and offers an insight into the biological processes causing variation in the genetically based trait. The predicted gene function analysis was based on gene cards, i.e. an integrated database of human genes (http://www.genecards.org). In Limousine breeds, differentially expressed predicted genes belong to the immunological and methyl transferase activities. However, for the Hereford breed, the predicted genes belong to the wide range of biological activities, for example: *ID3, C1QA, MAN1A2, SRP, SSH2, IRX1, GKAP1, KDM4C, NCK2, NPAS1 A1L539, TAGL, DDN1, SELK, COL24A1, Q95M07*, and *5NTC*. In Limousine

breed, the predicted gene transcripts were identified on BTA11 and BTA17. However, in Hereford breed, the predicted gene transcripts were identified on BTA2, BTA3, BTA5, BTA8, BTA9, BTA11, BTA12, BTA15, BTA16, BTA18, BTA19, BTA20, BTA21, BTA22, BTA23, and BTA26 (Table 4).

Validation of expression patterns of representative predicted gene sequences in selected cattle breeds by qRT-PCR analysis

Gene expression levels of target genes *EHMT1* (Limousine) and *NCK2* (Hereford) were verified by real time quantitative PCR (qRT PCR). After normalization of gene expression levels of both target genes with *GAPDH* reference genes in qRT-PCR, the efficiencies of the amplification of particular target transcripts were observed as: bovine *GAPDH* – 1.8, bovine *EHMT1* –2.05, and bovine *NCK2* – 1.6, respectively. These predicted target genes were chosen as they might represent the QTL regions for bovine body weight (McClure et al., 2010), with a preference for genes possibly involved in bovine postnatal growth and developmental processes.

The qRT-PCR analysis of within breed showed relatively differential levels of *EHMT1* and *NCK2* expressions at the age of 6, 9, and 12 months in investigated beef cattle. The age specific high expression levels of *EHMT1* were observed at the
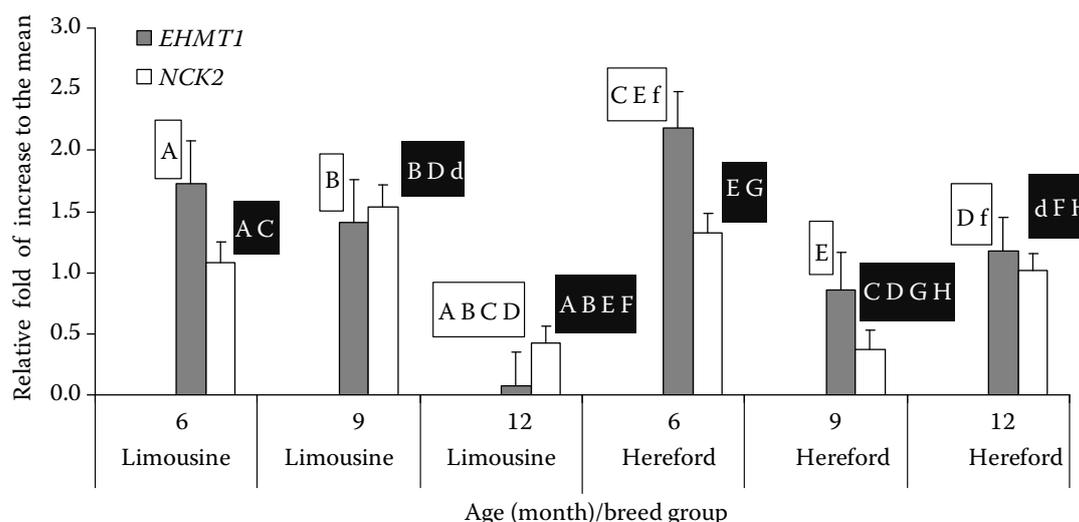

Figure 2. Within and between breed gene expressions levels of beef breed specific predicted target genes

LS means with the same letter are significantly different at $P < 0.05$ (small letters) and $P < 0.01$ (capital letters)

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Table 4. Identification of predicted gene transcripts expressed in pituitary gland tissue of young growing bulls from Limousine and Hereford breeds

Breed	TDF length	%ID	e-values	Predicted genes location at Btau_4.0	Contigs nearest to predicted genes	Gene index	Predicted genes	Gene symbol	Ensembl location at Btau_4.0
Lim	587	70	0.0056	16995464: 16996083	AAFC030 81535	gi|194674499	interleukin-15 precursor	IL15_BOVIN	chromosome 17: 17,109,757–17,125,718
Lim	174	88	0.0014	12579093: 12579713	AAFC030 50440	gi|194679425	small nucleolar RNA, H/ACA box 61	SNORA61	chromosome 28: 12,836,458–12,837,438
Lim	190	92	2.4e-08	109991043: 109991671	AAFC030 50497	gi|119904374	euchromatin histone lysine *N*-methyl-transferase	EHMT1	chromosome 11: 109,998,734–110,062,933
Her	589	76	0.00036	133670471: 133671092	AAFC030 58820	gi|112119494	inhibitor of DNA binding 3	ID3_BOVIN	chromosome 2: 133,738,693–133,740,311
Her	179	93	9.2e-05	134758870: 134759492	AAFC030 27915	gi|194665041	complement C1q subcomponent subunit A	C1QA_BOVIN	chromosome 2: 134,780,920–134,808,297
Her	277	86	0.0014	27896035: 27896655	AAFC030 82145	gi|194665483	mannosyl-oligosaccharide 1,2-alpha-mannosidase IB	MAN1A2	chromosome 3: 28,011,770–28,183,546
Her	295	84	0.0056	22835147: 22835766	AAFC031 04903	gi|194666713	WDR51B protein	A5PKH9_BO-VIN	chromosome 5: 21,893,860–22,053,189
Her	247	91	0.0056	55116310: 55116929	AAFC030 02886	gi|194671482	eukaryotic type signal recognition particle RNA	SRP_euk_arch	chromosome 11: 55,309,409–55,309,700
Her	241	93	0.00036	67391247: 67391868	AAFC030 77346	gi|119904842	glypican 6	A1L539_BOVIN	chromosome 12: 67,849,931–67,870,468
Her	161	90	2.3e-05	26319598: 26320221	AAFC030 44160	gi|194673254	transgelin	TAGL_BOVIN	chromosome 15: 26,321,331–26,326,591
Her	524	77	0.0014	21078306: 21078926	AAFC031 08749	gi|194675875	slingshot homolog 2 (Drosophila)	SSH2	chromosome 19: 21,184,223–21,226,291
Her	210	88	0.0056	73268376: 73268995	AAFC030 56209	gi|119913433	iroquois homeobox 1	IRX1	chromosome 20: 73,284,676–73,289,502
Her	209	83	0.0056	34845565: 34846184	AAFC031 15243	gi|194676879	duodenase-1	DDN1_BOVIN	chromosome 21: 34,853,147–34,855,206
Her	202	84	2.3e-05	47995909: 47996532	AAFC030 24159	gi|194677336	selenoprotein K	SELK_BOVIN	chromosome 22: 48,071,746–48,074,059
Her	399	83	5.9e-06	3225676: 3226300	AAFC030 19321	gi|194677546	BEN domain-containing protein 6	BEND6	chromosome 23: 3,333,492–3,375,568
Her	371	92	0.0056	132809890: 132810509	AAFC030 18263	gi|194665041	UPF0490 protein chromosome 1 open reading frame 201 ortholog	CA201_BOVIN	chromosome 2: 132,791,427–133,003,584
Her	508	77	0.0014	61564214: 61564834	AAFC030 83156	gi|194665661	alpha 1 type XXIV collagen	COL24A1	chromosome 3: 61,522,442–61,877,592
Her	517	69	0.00036	81163419: 81164040	AAFC030 27414	gi|194669609	G kinase-anchoring protein 1	GKAP1_BOVIN	chromosome 8: 81,169,085–81,259,091

Table 4 to be continued

Breed	TDF length	%ID	e-values	Predicted genes location at Btau_4.0	Contigs nearest to predicted genes	Gene index	Predicted genes	Gene symbol	Ensembl location at Btau_4.0
Her	191	95	0.0056	39847272: 39847891	AAFC031 23942	gi|119900320	lysine (K)-specific demethylase 4C (KDM4C)	LOC535671	chromosome 8: 39,815,936-40,008,364
Her	238	85	9.6e-08	101505819: 101506446	AAFC030 91350	gi|119901745	PARKIN (ubiquin E3 ligase PRKN)	Q95M07_BO-VIN	chromosome 9: 101,382,096-101,928,910
Her	262	86	6.2e-09	47626345: 47626974	AAFC030 85598	gi|119903614	NCK adaptor protein 2	NCK2	chromosome 11: 47,625,137-47,674,398
Her	514	69	9.2e-05	3244387: 3245009	AAFC030 22833	gi|194673974	SLIT-ROBO Rho GTPase activating protein 2	SRGAP2	chromosome 16: 3,009,452-3,266,742
Her	225	87	0.0056	53877226: 53877845	AAFC030 46945	gi|194675318	neuronal PAS domain protein 1	NPAS1	chromosome 18: 53,876,672-53,892,486
Her	170	85	2.3e-05	24139926: 24140549	AAFC031 05927	gi|194679004	cytosolic purine 5'-nucleotidase	5NTC_BOVIN	chromosome 26: 24,092,572-24,165,911

TDF = transcript derived fragments, Her = Hereford, Lim = Limousine

age of 6 months in both Limousine and Hereford breeds (Figure 2). However, the expression level of *NCK2* was the highest at the age of 6 months in Hereford and at 9 months in Limousine. Further, within Limousine breed expression levels of *EHMT1* and *NCK2* were the lowest at the age of 12 months (Figure 2). Whereas, within Hereford breed, the expression levels of both *EHMT1* and *NCK2* were the lowest at the age of 9 months. In-between breed qRT-PCR analysis, the designed breed specific predicted target genes (*NCK2* and *EHMT1*) showed comparatively higher expression levels at the age of 6 and 12 months in Hereford breed and at the age of 6 and 9 months in Limousine breed (Figure 2).

DISCUSSION

Genome-wide expression analysis is rapidly becoming an essential tool for identifying and analyzing genes controlling or involved in biological processes. In cattle, breed specific gene expression profiling was first successfully conducted by investigating milk and meat type of cattle breeds in context to metabolic traits (Dorroch et al., 2001). They utilized DD RT-PCR (Liang and Pardee, 1992) differential display technique and identified 55 ESTs markers for metabolic traits. However, in other tissues specific gene expression profiling in context to nutrient utilization, 161 and 116 ESTs for liver and intestine tissues were identified in growing Charolais and German Holstein bulls using qRT-PCR (Schwerin et al., 2006). In a recent study, the gene expression profiling of pituitary gland tissue was investigated in two dairy cattle breeds and identified 12 and 4 differentially expressed (DE) predicted genes in Polish HF and Polish Red cattle (Pareek et al., 2012), as compared to the presented results which identified 3 and 21 DE predicted genes for Limousine and Hereford breeds. Furthermore, by using qRT-PCR assay, gene expression levels of two breed specific predictive target genes, i.e. Glycophorin C (Polish HF) mapped on BTA2 and ALOX5 isoform 1 (Polish Red) mapped on BTA28 were measured and validated (Pareek et al., 2012). While, in the present study, the validated *EHMT1* (Limousine) and *NCK2* (Hereford) predicted genes both were mapped on BTA11. In general, each breed specific target genes representing both dairy breeds (Pareek et al., 2012) and beef breeds (present study) have shown significant differences

in the gene expression levels at both within breed (developmental ages) and between breed (dairy and beef breeds).

In cattle, both identified DE predicted genes were not studied extensively in context to economic traits. In general, the EHMTs are a family of evolutionarily conserved proteins, responsible for the epigenetic code activities through methylation of histone 3 at lysine 9 (H3K9) (Stabell et al., 2006). In mammals, two *EHMT* paralogs exist, i.e. *EHMT1*/glycerol 3-phosphate permease (GLP) and *EHMT2*/G9a. The EHMT1 protein plays an important role in dynamic regulation of histone modifications and eukaryotic gene transcription, which are critical during development. However, little is known about how their activities are coordinated *in vivo* to regulate specific biological processes (Di Stefano et al., 2011). In humans, a nonsense mutation and a frame-shift mutation within the *EHMT1* gene causes 9q subtelomeric deletion syndrome (Kleefstra et al., 2006). Another validated predicted gene *NCK2* is an adaptor protein, which is mainly implicated in binding and recruitment of various proteins involved in the regulation of protein tyrosine kinases receptor. It is through these regulatory activities that this protein is believed to be involved in cytoskeletal reorganization. In cattle, expression of this gene has not been defined and well-studied, however, several studies were provided as it is potentially linked to the regulation of cellular actin (Bladt et al., 2003; Stylli et al., 2009), cell movement (Rivera et al., 2006), skeletal muscle differentiation/regeneration and cytoskeleton remodelling (Xu and Henkemeyer, 2009; Gehmlich et al., 2010). Among non-validated predicted genes, several predicted functional genes were identified for Limousine (*n* = 2) and Hereford (*n* = 20) breeds. Based on their biological functions of identified predictive genes, one can suggest *A1L539* (cell surface growth factor receptor), *SSH2* (regulation of actin filament dynamics), *COL24A1* (regulation of fibrillogenesis), *GKAP1*, and *NPAS1* (germ cell development) as potential candidate genes for bovine body growth and developmental processes.

CONCLUSION

The cDNA-AFLP technique has successfully been utilized to *Bos taurus* transcriptome in determining the gene expression patterns as well as identifying predicted genes expressed in bovine pituitary gland tissue. Study revealed higher numbers of differentially expressed transcripts at an early stage of developmental life in Hereford (6 months) and later stage of developmental life in Limousine (12 months). In this study, we have obtained an overview of bovine pituitary transcriptome profile and identified 24 differentially expressed predicted transcripts similar to known bovine genes, which may play important roles in bovine postnatal growth and developmental processes. Finally, the study suggested that validated bovine *EHMT1* and *NCK2* genes can be considered as candidate genes for bovine postnatal growth and developmental processes.

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