Identification of differentially expressed genes for fescue toxicosis in high and low tolerant Angus cows

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Abstract

Background Fescue toxicosis (FT) is the multifaceted syndrome that causes the major loss of revenue in beef industry. Management of FT has been substantial challenge for the beef industry. Little research has been conducted to identify host genetic variation for FT response. Therefore, the objectives of this study were 1) to identify differentially expressed genes (DEG) in animals with contrasting response to fescue toxicosis, 2) to assess the biological relevance of DEG and 3) to investigate the relationships of DEG through gene networks in Angus cows. Results Genotype-by-location-by-time interaction was evident, with one location (2,296) having much greater number of DEG (q-value < 0.1) between HT and LT animals than the other (554). In addition, there was a greater number of DEG (q-value < 0.1) between HT and LT animals on week 5 (3,892) than on weeks 1(1,413), 9 (1,384), and 13 (573). So, further analyses focused on DEG between HT and LT animals on week 5 at the most toxic location. The most significant DEG between HT and LT animals had relevant functions associated with FT: cellular growth (SPDYC, HEYL, ANXA13), cardiovascular function (FGB, HBA, WNT11, BPIFB4, MESP2), protein metabolism (ENPP6, MMP8), and immune response (CTBS, SDC2, CXCL13, IL-13, JAKMIP2). The strongest positive partial correlation (0.99) was between CTBS and CXCL13, where CTBS is involved in carbohydrate metabolism and immune function, and CXCL13 is involved in immune, inflammatory, and defense response, and G-protein coupled pathway. The regulation of the most significant DEG between HT and LT animals on week 5 are highly correlated, indicating a complex interaction between. When all DEG were analyzed, the enriched biological processes associated with fescue toxicosis included immune response, cardiovascular function and development, metabolic, cellular and biological processes, and fertilization. Conclusions These findings provide potential biomarkers that should be evaluated for selection of cattle with greater tolerance to fescue toxicosis which will help to establish the herd with fescue tolerant cows in regions where high endophyte infected pasture is present.

Background

Fescue toxicosis (FT) is a disease in cattle caused by infected tall fescue pastures with fungal endophyte, *Neotyphodium coenophialum*. Cattle suffering from fescue toxicosis may have reduced pregnancy and calving rates, reduced hair shedding, heat stress, suppressed growth and appetite, and insufficient blood flow to the extremities [1] With these symptoms, fescue toxicosis causes loses of over $3.2 billion in the U.S. beef industry [2].

Management of FT has been substantial challenge for the beef industry. Research on methods to alleviate the impact of FT has mainly focused on two aspects: pasture management and animal management. Pasture management involves replacing infected tall fescue with non-infected or other grass species for grazing [3], diluting the endophyte infected tall fescue with other grasses, using the stockpiled tall fescue to prevent seed head formation [4]. Animal management consists of multiple strategies that range from nutritional supplementation of various or increasing protein levels in the diet to selection of traits which make the animals most susceptible or resistant to the fescue toxicosis. Spring-calving cows had greater calving rate in comparison to fall-calving [5].

On the host genetics side, little research has been conducted to identify variation for response to FT. Browning [6] reported no difference in cortisol and prolactin concentration, respiration rate, rectal temperature, skin temperature between Hereford and Red Brahman steers injected with ergotamine. A second study of Browning [7] mentioned reduced susceptibility of heat stress of Senepol steers than Hereford steers when fed with endophyte infected diet. Gould and Hohenboken [8] reported particular Hereford bull sired calves were resistant to fescue toxicosis in comparison to progenies of another sire. Moderate heritability (0.35) for the first month of hair shedding in Angus cattle under toxic fescue was reported by Gray et al. [9], with this trait having negative genetic correlation (-0.58) with weaning weight. Using genetic markers, [10] reported the association between a SNP within the dopamine receptor D2 (*DRD2*) gene and prolactin response in beef cattle grazing toxic tall fescue. Additionally, Bastin et al. [11] reported the relationship of XK, Kell blood group complex subunit-related family, member 4 (*XKR4*) genotype and circulating prolactin in beef cattle grazed in toxic tall fescue. All of the previous studies reported suggested that genetic components of fescue toxicity exist. More recently, a commercially available test called T-Snip was established by AgBotanica, LLC (Colombia, MO). Masiero et al. [12] found the significant correlation between dam T-Snip genotypes and calf weaning weight, whereas Galliou et al. (unpublished results) found significant association between T-Snip genotypes with cow body weight during pregnancy and 205-d adjusted calf weaning weight.

In addition to using genetic markers, few studies have evaluated the transcriptomic patterns of cattle under FT [13–15]. However, these studies focused on the identification of genes differentially expressed between animals fed or not toxic fescue. For genetic selection purposes, the focus should be on within breed differences with the objective of identifying genes that have different expression patterns between, for example, tolerant and susceptible cattle. Therefore, this major research component is currently missing in the literature. Thus, the objectives of this study were 1) to identify differentially expressed genes (DEG) in animals with contrasting response to FT, 2) to assess the biological relevance of DEG, and (3) to investigate the relationships of DEG through gene networks in Angus cows.

Methods

The study was conducted in compliance with all welfare regulations, with all the study procedures being approved by the Institutional Animal Care and Use committee of North Carolina State University (protocol #13-093-A). After the study, all animals returned to the research herd.

Study location, animals, and sampling

Purebred, multiparous (parities 2 to 4), pregnant Angus cows (n = 149) were taken from two beef herds from North Carolina: Butner Beef Cattle Field Laboratory (BBCFL; Bahama, NC, USA) and Upper Piedmont Research Station (UPRS; Piedmont, NC, USA). There were 71 and 78 cows at BBCFL and UPRS, respectively. Farms were located approximately 100 km away from one another. Animal had free access to water and tall fescue pasture. Half of the tall fescue pasture in BBCFL was infected with 95%

endophyte and the other half was infected with 65% endophyte. At UPRS 95% of the tall fescue pasture was infected with endophyte. Weekly body weight (BW) data were collected on all animals for 13 weeks [from April 26 (week 1) to July 19 (week 13), 2016]. Blood samples (10 ml) of each cow were collected from all cows via jugular venipuncture into Tempus Blood RNA Tubes (Life Technologies, Carlsbad, CA, USA) on weeks 1, 5, 9, and 13 for subsequent gene expression analysis.

Identification of animals with high and low tolerance to FT

Out of 149 cows, 40 cows showing extreme growth performance were selected for further RNA-seq analysis. For each animal, growth during the trial was estimated as the slope of regression analysis of BW on weeks (average weekly gain; AWG). Slopes (i.e. AWG) were estimated based on 3 window periods: weeks 1 through 13 (w1_13), weeks 1 through 7 (w1_7), and weeks 7 through 13 (w7_13) to assess the effect of increase in temperature from April to July, availability of forage, and exposure of infected tall fescue. The AWG data for each of these scenarios were analyzed using the following model:

$$A W G i j k = \mu + L i + P j + b 1 (i B W k - i B \hat{W}) + e i j k \quad [Eq. 1]$$

where A W G i j k is the AWG of the cow; μ is intercept; L i is the fixed effect of the i^{th} location; P j is the fixed effect of the j^{th} parity; b 1 is the partial regression coefficient for the covariate of initial BW (*iBW*); i B W k is the iBW of the k^{th} cow; and e i j k is the residual associated with y i j k , with e i j k ~ N 0 , I σ e 2 where *I* is the identity matrix. Statistical analysis was performed in SAS 9.4 (Statistical Analysis System, Cary, NC, USA).

Identification of animals with high (HT) or low (LT) tolerance to FT were based on the residuals from Eq. 1. The top (positive) 20 and bottom (negative) 20 residuals, with equal representation from each location (i.e. 20 from each location), were classified as HT and LT, respectively, for a total of 40 selected animals. This was done for each of the 3 windows periods, which resulted in different sets of selected animals, depending on the window period. In order to identify which of the 3 periods better expressed the impact of FT on performance, two additional analyses were performed. First, the additive genetic variance (σ a 2) of the data for each of the window periods were estimated with the following model:

$$A W G i j k = \mu + L i + P j + b 1 (i B W k - i B \hat{W}) + a i j k + e i j k \quad [Eq. 2]$$

where A W G i j k , μ, L i , P j , b 1 , i B W k , and e i j k are as previously defined in Eq. 1; and a i j k is the random animal effect, with a k ~ N 0 , A σ a 2 where *A* is the additive pedigree matrix including 2,531 animals. Analysis was performed in ASReml v.4 [16]. The estimated σ a 2 of each window period (w1_7, w1_13, and w7_13) was compared between each other and tested using an *F*-test. In addition, the AWG residuals (AWG_res) of the selected animals based on Eq. 1 were analyzed with the following model:

$$A W G \_ r e s i j k = \mu + G i + L j + W k + i n t e r a c t i o n s + e i j k \quad [Eq. 3]$$

where μ and e i j k are as previously defined; A W G _ r e s i j k is the AWG_res of the selected animal; G i is the fixed effect of the i^{th} genetic group (HT or LT); L is the fixed effect of the j^{th} location (BBFCL or UPRS); W k is fixed effect of the k^{th} window period (w1_7, w1_13, or w7_13); and *interactions* represent all possible interactions between these effects. There were no significant effects (*P*-value ≥ 0.159) for the main effects of *W* and *L*, and for the interactions of *G*L*W*, *L*W*, and *G*L*. There was a significant (*P*-value < 0.0001) interaction between *G* and *W*, and the main effect of *G*. Statistical analysis was performed in SAS 9.4 (Statistical Analysis System Institute, Inc., Cary, NC, USA).

The estimated σ a 2 for each window period and for each G by window period are presented in Table 1. The estimated σ a 2 for w1_7 [2.22 $(kg/week)^2$] was greater (*P* < 0.01) than for w1_13 [0.58 $(kg/week)^2$] and w7_13 [1.04 $(kg/week)^2$]. In addition, LT animals for w1_7 (-2.34 kg/week) had lower (*P* < 0.01) AWG_res than other LT animals for w1_13 (-1.39 kg/week) and w7_13 (-1.73 kg/week). Similarly, HT animals for w1_7 (2.42 kg/week) had greater (*P* < 0.01) AWG_res than other HT animals for w1_13 (1.78 kg/week) and w7_13 (1. 63 kg/week). Because of the greater σ a 2 and the more extreme AWG_res values, data using w1_7 was used for subsequent analysis.

RNA extraction, sequencing, and bioinformatics

Total RNA from the 40 selected cows (w1_7) was extracted by Tempus™ RNA isolation kit (Applied Biosystems, Foster City, CA, USA. The RNA quantity and quality were determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). All the samples were sent to the Genomic Sciences Laboratory (North Carolina State Laboratory, Raleigh, NC, USA) for library construction and RNA-sequencing. Sequencing was performed in an Illumina NextSeq 500 instrument (Illumina, Inc. San Diego, USA), generating 150 bp paired-end reads. A total of 10 flow cells were used with 16 samples in each flow cell. The samples were balanced by the time of sample taken, genetic group, and location.

The quality of raw reads was evaluated with FastQC [17]. Sequence reads for each sample were mapped to *Bos taurus* UMD3.1.88 reference genome using Bowtie2 [18]. The number of counts for each sample was obtained with *Subread* package from SourceForge [19]. A total of 3,158,121,718 paired ends were generated with an average of 19,738,261 reads/sample. Reads were mapped to 81.25% (20,000) from total 24,616 genes annotated in the Bos Taurus reference UMD3.1. Genes with total of counts less than 4 times the number of samples in each combination of genetic group, location, and time were eliminated to avoid low counts. After quality control, 15,360 genes were used for the analyses. Normalized factors were calculated with the *TCC* package [20] in R (R Development Core Team; Vienna, Austria), using the trimmed mean of M values (TMM) methodology to obtain the normalized library sizes for each sample for subsequent statistical analysis.

Statistical Analysis

Gene expression data were analyzed with the following negative binomial model:

$$Count_{ijklm} = \mu + G_i + T_j + L_k + interactions + C_l + b_1 R_{ijklm} - \hat{R} + \log S_{ijklm} + e_{ijklm} \quad \text{[Eq. 4]}$$

where $Count_{ijklm}$ is the number of raw counts; μ is intercept; G_i is the fixed effect of i^{th} genetic group (HT or LT); T_j is the fixed effect of j^{th} time (week 1, 5, 9, and 13); $L_k$is the fixed effect of k^{th} location (BBFCL or UPRS); *interactions* represent all possible interactions between these effects; C_l is the fixed effect of l^{th} flow cell (10 flow cells); $R_m$is the covariate of m^{th} RNA integrity factor; S_{ijklm} is the TMM-normalized library size used as an offset; and e_{ijklm} is the residual associated with $Count_{ijklm}$. Analyses were performed using three covariance structures for residuals: first-order autoregressive, compound symmetry, and independent. Three models were tested for each of the 15,360 genes, and final model was selected based on the Akaike Information Criterion (AIC). The dispersion parameter of the model was calculated for each gene, squeezing it towards a global dispersion, using the package *edgeR* [21] in R.

False-discovery rate (FDR) [22] was used to adjust *P*-values (*q*-values). Significant DEG were identified at *q*-value < 0.1 for effects of interest. All the data were analyzed using the *GLIMMIX* procedure of SAS 9.4 (Statistical Analysis System Institute, Inc., Cary, NC, USA).

Functional Annotation Analysis

The enrichment of Gene Ontology (GO) terms associated with DEGs were analyzed using PANTHER Enrichment Analysis [23]. The *Bos taurus* genome that were mapped in this study was used as the reference list. Biological, molecular, and cellular functions were considered significant at *P*-value < 0.05 for difference of genetic groups on week 5 at UPRS.

Correlation network

Gene network analysis was done by using the top 15 DEG in order to evaluate the relationship among the most impacting DEG. Connections between genes (i.e. nodes) were included when a pair of genes showed a partial correlation greater than |0.5|. The correlation matrix and gene-networks were constructed using *ppcor* [24] and *qgraph* [25] packages in R. The data was pre-adjusted for the fixed effects of genetic group, genetic group-by-time and genetic group-by-location prior to analysis.

Results

Differentially Expressed Genes

A total of 592, 1,082, 3753, 88, 3,923, 2,587, and 924 DEG (*P*-value < 0.1) were identified for the effects of genetic group, location, time, genetic group-by-time, location-by-time, location-by-genetic group, and genetic group-by-location-by-time interaction, respectively (Fig. 1A). Within BBCFL and UPRS, 554 and 2,296 DEG (*P*-value < 0.1) were identified for effect of genetic group, respectively, with only 90 DEG overlapping between locations (Fig. 1B). Because of the much greater number of DEG between genetic groups at UPRS compared to BBCFL, further results will be focused on this location. Again, there were more DEG (*P*-value < 0.1) between genetic groups on week 5 (3,892) compared to all other weeks: 1,413, 1,384, 573, for weeks 1, 9, and 13, respectively (Fig. 1C), indicating that there is a timing/seasonal effect playing a role in the regulation of genes in response to FT.

The volcano plots for the effect of genetic group-by-week at UPRS are given in Fig 2. A much greater number of DEG between genetic groups were found on week 5. Out of the 924 DEG that were identified for genetic group-by-location-time interaction (*P*-value < 0.1), there were (*q*-value < 0.1) 59 and 253 DEG between genetic groups at UPRS, for weeks 1 and 5, respectively. No DEG were identified (*q*- value < 0.1) between genetic groups at UPRS, for weeks 9 and 13. In addition, visually, there were more genes being upregulated in LT animals compared to HT, especially on week 5 (Fig 2B). Thus, further analyses focused on DEG between genetic groups at UPRS on week 5.

Major Differentially expressed genes on week 5 at UPRS

In our study, the top 10 most significant up- and down- regulated DEG for the effect of genetic group (HT compared to LT) on week 5 at UPRS are summarized in Table 2. The complete list of DEG (*q*-value < 0.10) for the effect of genetic group on week 5 at UPRS is provided in supplemental file 1. Results are presented as the $\log_2$ fold change (FC) between LT and HT groups, with negative and positive values representing upregulation of genes in LT and HT animals, respectively. Of these genes, the most significant upregulated genes in LT and HT animals were ectonucleotide pyrophosphatase/phosphodiesterase 6 (*ENPP6*) and Mesoderm posterior bHLH transcription factor 2 (*MESP2*), respectively, with $\log_2$FC of -1.90 [95% confidence interval = -2.92, -0.89] (*q*-value = 0.005) and 0.91 [0.35, 1.47] (*q*-value = 0.01), respectively. The largest |$\log_2$FC| was observed for Chitiobiase (*CTBS*), which was upregulated in LT animals compared to HT; $\log_2$FC = -3.13[-4.88, -1.38] (*q*-value = 0.008).

Gene network

The partial correlation network using the 20 most significant DEG (*q*-value < 0.10) between genetic groups at UPRS on week 5 (Table 2) is presented in Figure 3. On average, each gene was connected to 10.6 genes. The strongest positive partial correlation (0.99) was between *CTBS* and *CXCL13*, where, *CTBS* is involved in carbohydrate metabolism and immune function, and *CXCL13* is involved in immune,

inflammatory, and defense response, and G-protein coupled pathway. The strongest negative partial correlation (-0.96) was between *CTBS* and *SDC2*, where *SDC2* is predominantly involved in nervous system development, dendrite development and acts as co-receptor of enzymes. *ENPP6* had the greatest number of connections (15) between genes, with most (9) of connections being negative. This gene was up-regulated in LT animals. In contrast, *ENSBTAG00000047030* had the least number of connections, being negatively partially correlated with *ENPP6* only. Based on this analysis, other important hub genes include *MMP8* (14 connections), *SDC2* (14 connections), and WNT11 (14 connections).

Enrichment analysis

The enrichment analysis of genes was conducted using the 4,453 DEG (*q*-value < 0.10) for the effect of genetic group on week 5 at UPRS. The top 20 biological processes and GO terms that were enriched (*P*-value < 0.05) are presented in Table 3. The complete result of enrichment analysis (*P*-value < 0.05) for the effect of genetic group at week 5 in UPRS is provided in supplemental file 2. Overall, the enrichment analysis showed that DEG have general functions, such as metabolic, biosynthetic, and catabolic processes, as well as DNA and RNA-related functions, such as translation, transcription, and repair. Among all GO terms in Table 3, the most common genes were valyl-tRNA synthetase 2 (*VARS2*), leucine rich repeat containing 47 (*LRRC47*), arginyl-tRNA synthetase 2 (*RARS2*), aspartyl-tRNA synthetase (*DARS*), phenyl alanyl-tRNA synthetase subunit alpha (*FARSA*), exosome component 9 (*EXOSC9*), poly (A) specific ribonuclease subunit (*PAN2*), and leucyl-tRNA synthetase 2 (*LARS2*).

Discussions

Previous research has been conducted to evaluate the effect of fescue toxicosis on gene expression and their pathways in beef cattle [13–15]. These studies focused on the identification of DEG between animals fed or not toxic fescue. However, in our study, we focused on within breed difference for the identification of DEG. The DEG identified from all of these three studies have functions associated with cellular development, cell-mediated immune response, hematological system development and hematopoiesis, growth hormone signaling and metabolism which was in concordant with this study.

The high number of DEG between genetic groups at UPRS was in accordance with the results presented by Mayberry et al. [26] using the same animals and locations. These authors reported genetic group-by-location effect for several phenotypes (e.g. hair shedding, rectal temperature, prolactin, etc.) with much greater differences between genetic groups at UPRS compared to BBCFL. These differences between genetic groups-by-location must be due to the higher fescue infection rates at UPRS compared to BBCFL [26].

Most Differentially expressed genes on week 5 at UPRS

The upregulated genes for LT and HT animals could be broadly grouped into immune response, blood, and cardiovascular function, development, cellular, and biological processes, and fertilization. Of all these genes, we identified seven genes involved in immune functions: *CTBS*, Syndecan 2 (*SDC2*), Matrix metallopeptidase (*MMP8*), C-X-C motif chemokine ligand 13 (*CXCL13*), Interleukin-13 (*IL-13*), and Janus kinase and microtubule interacting protein 2 (*JAKMIP2*). We identified seven DEG involved in blood and cardiovascular function: Fibrinogen beta chain (*FGB*), Hemoglobin subunit alpha (*HBA*), *ENSBTAG00000035224*, Mesoderm posterior bHLH transcription factor 2 (*MESP2*), Wnt family member 11 (*WNT11*), BPI fold containing family B member (*BPIFB4*), and *CXCL13*. Histone H2B type 1 (*ENSBTAG00000024188*) is involved in fertilization. Claudin 19 (CLDN19) and Oligodendrocyte transcription factor 1 (*OLIG1*) were identified to have neuronal functions. Other identified DEG have more general functions, such as cellular differentiation, biological process and apoptosis: Speedy/RINGO cell cycle regulator family member C (*SPDYC*), Hes related family bHLH transcription factor with YRPW motif-like (*HEYL*), Ectonucleotide pyrophosphatase/phosphodiesterase 6 (*ENPP6*), Annexin A13 (*ANXA13*), *ENSBTAG00000047030,* and *SDC2*. Among all genes, *CTBS*, *CLDN19*, *SPDYC*, *HEYL*, *ENPP6*, *SDC2*, *MMP8*, *ENSBTAG00000035224, FGB,* and *HBA* were upregulated in LT animals whereas *WNT11*, *BPIFB4*, *ENSBTAG00000047030, JAKMIP2*, *MESP2*, *OLIG1*, *IL-13*, *ANXA13*, *ENSBTAG00000024188,* and *CXCL13* were upregulated in HT animals. *CTBS* has chitin binding and hydrolase functions [27], whereas chitin induces cytokine production, leukocyte recruitment, and alternative macrophage activation [28]. Chitin is sensed by the immune system as a pathogen associated molecular pattern (PAMP) through specific membrane-bound receptors and plays a key role in defense against pathogens [29]. This could be related to the loss of immune function in cows with fescue toxicosis and hence increase in chitin binding genes. *SDC2* has immune function and acts as co-receptor for enzymes and cytokines. These are found to control adhesion and migration of cells during development, wound healing, angiogenesis, infection and inflammation [30]. Settivari et al. [31] also found the upregulation of *SDC2* in rats with endophyte diet. Polymorphonuclear neutrophils are produced by *MMP8*, which are released at site of inflammation [32]. *MMP8* is essential for lipopolysaccharide-induced inflammatory response and chemokine production and is an indicator of neutrophil function [33]. *CXCL13* is made primarily by stromal cells and follicular dendritic cells in B cell follicles, and recruits both B cells and CD4+ T follicular helper cell. *CXCL13* is an important target molecule for the regulation of B-cell trafficking to secondary lymphoid tissues [34]. *CXCL3* is found in lymphoid organ that develop in the inflamed meninges of humans with multiple sclerosis and autoimmune encephalomyelitis in mice [35]. *IL-13* is produced by helper T lymphocytes and mediates the effect of several immune cells. It has major role in immune function [36, 37]. *IL-13* also enhances the antigen presentation by macrophages, which increases the Th1 type responses generated in response to intracellular pathogens [38]. Although there were differences in *IL-13* expression (q-value = 0.028) between genetic groups in our study, [26] did not find differences in serum IL-13 levels between LT and HT animals. Thus, this difference in expression might be related to regulation of other genes and/or pathways. For example, IL-13 is interacts with Janus kinase 2, which has major role in immune function [39, 40] and prolactin [41]. *JAKMIP2* is involved in Janus kinase and microtubule binding [42]. The up-regulation of Janus kinase 2 serves as the signaling molecule for prolactin receptor [41] which helps to increase the serum prolactin, which is an indicator of FT tolerance in cattle [43, 44]. Although we

identified major DEG between genetic groups with direct immune function, little is known about the immune response to FT. Settivari et al. [31] demonstrated that mice with ergot alkaloid exposure have increased liver-specific enzymes and speculated that this could be due to the hepatic inflammation due to the alkaloid exposure. In addition, Saker et al. [45] reported the decreased immune system is associated with grazing tall fescue. There was decrease in T cell derivatives which caused the immune-suppressive effect due to exposure to ergot alkaloids [46]. Therefore, we could speculate that these 6 identified DEG may have important roles in determining the levels of tolerance to FT in beef cattle.

Vasoconstriction is one of the major symptoms of FT [47]. Numerous studies [47, 48] reported that animals grazing on endophyte infected tall fescue or consume diet with ergot alkaloid have vasoconstriction on the peripheral parts. The FGB protein enhances blood coagulation [49], which narrows blood vessels through contraction of smooth muscle cells of blood vessels. *HBA* helps in transporting of oxygen and formation of heme content of hemoglobin [50]. *ENSBTAG00000035224* is novel gene found in extracellular space and has serine-type endopeptidase inhibitor activity [42]. This serine-type endopeptidase inhibitor activity helps in prevention of formation of blood clot and might be associated with coagulopathy conditions in cattle with fescue toxicosis. *MESP2* is essential for the development of cardiac mesoderm, heart morphogenesis and embryonic pattern specification [51]. *BPIFB4* stimulates the high blood pressure via impairment of nitric oxide signaling [52]. Nitric oxide is related to reduced pressure to periphery but increased blood pressure towards central part [53]. Reduced blood to the periphery might cause hypoxemia resulting in subsequent increase in respiration rates, which is a known symptom in cattle experiencing FT [54]. In humans, Vecchione et al. [52] found that *BPIFB4* is a powerful enhancer for endothelial vasorelaxation and revascularization, further supporting its potential role in FT. *WNT11* has several functions, such as promotion of cardiomyocyte differentiation, activation of kinase activity, and osteoblast maturation [55]. *WNT11* modulates the inflammation by bacterial invasion in myocarditis heart and improves the recovery after myocardial injury [56]. In addition to having role in immune responses, *CXCL13* is also involved in G-protein coupled pathway. The G-protein coupled receptor protein is associated with congestive heart failure, which is stimulated during period of stress [57, 58]. With the congestive heart failure, there could be irregularities in blood circulation which is the major clinical symptoms of fescue toxicity in cattle. In agreement with the present study that identified DEG related to blood and cardiovascular system, previous studies [48, 59] also reported that cattle consuming high endophyte diet have reduction in blood flow to the extremities and coagulopathy conditions when compared to the steers consuming low-endophyte diet.

SPDYC is involved in protein kinase binding. This family of protein enhances the meiotic maturation, increases cell proliferation in mammalian cells and promotion of cell survival by decreasing the apoptosis of cells with DNA damaging events [60]. *HEYL* genes are involved in skeletal muscle and neuronal cell differentiation, and cardiac cell morphogenesis. Previous studies [61, 62] showed that *HEYL* promotes the differentiation of neural progenitor cells and it is the key regulator of embryonic development or differentiation in different species. *ENPP6* is highly expressed in liver sinusoidal epithelial cells and developing oligodendrocytes, which is a choline specific phosphodiesterase that hydrolyzes the glycerophosphocholine that participate in choline metabolism, which is major component of myelin

sheath [63]. The high expression of *ENPP6* might be related to the defense of body towards the impairment of myelin sheath. This impairment of myelin structure is related to convulsions and seizures [64], which are the major symptoms fescue toxicosis. In addition to immune-related functions, *SDC2* interacts with the adhesion molecules, growth factors, and other effector system that support in shaping, maintenance, and repair of an organs [65], whereas their expression occurs in a cell- and development-specific pattern [66]. These are found to control cell proliferation, differentiation, adhesion, and migration during development, wound healing, angiogenesis, infection, and inflammation [30]. *ANXA13* is highly tissue-specific, being expressed only in intestinal and kidney epithelial cells *ANXA13* helps in calcium-dependent phospholipid-binding protein family that plays a role in the regulation of cellular growth and in signal transduction pathways [67, 68]. The upregulation of genes associated with cell growth, repair, proliferation, and differentiation could be related to loss of body condition, as a result of reduced weight gain in cows consuming the endophyte-infected tall fescue.

Among the genes presented in Table 2, Histone H2B type 1 (*ENSBTAG00000024188*) is involved in fertilization. Histone protein plays major role in development of an oocyte during which an oocyte acquires a specialized extracellular matrix and synthesizes a unique set of proteins to become a fertilizable egg [69]. Differential expression of histone H2B in association with H2A has been identified in the oocyte and they are correlated with genes in cumulus cells (FSHR, EGFR, and GHR) of oocyte as an indicator of oocyte competence [70] to get fertilized. Overall, the major DEG identified between HT and LT animals in our study indicate that genes associated with tolerance to FT are associated with the major symptoms of fescue toxicosis: loss of immune function, vasoconstriction, increased respiration rate and blood pressures, loss of fertilization, reduced growth, and nervous system.

Gene network

The gene network for 20 major genes (Figure 3) indicated that the expression patterns of the most significant genes (Table 2), was simultaneously up- or down-regulated in the same tolerance group. Altogether, these results indicate that the regulation of the most significant DEG (Table 2) between LT and HT animals on week 5 at UPRS are highly correlated, indicating a complex interaction between genes that might help explaining phenotypic differences between HT and LT animals.

Enrichment analysis

To date, there are very few studies evaluating the transcriptome of animals under FT. Using blood transcriptome from cattle fed pastures with or with toxic fescue, Atchley [15] identified *protein folding* as the only biological process enriched in the study. Liao et al. [13] found DEG involved in cell mediated immune response pathways when evaluating liver tissue in cattle grazing high or low levels of toxic fescue. Additionally, Li et al. [14] identified DEG by microarray analysis of pituitary samples of steers grazed in pasture with high versus low endophyte-infected fescue, involved in pathways of cardiac

hypertrophy signaling, axonal guidance signaling, growth hormone signaling, eNOS signaling, dopamine receptor signaling and prolactin signaling. Results from these three studies were very different from out study. In addition to differences between tissues evaluated, another major difference between results presented in the literature and ours is the fact that our study focused on identifying DEG and biological processes within a breed; in other words, between animals showing contrasting responses to FT. In contrast, biological processes and DEG reported in the literature are between cattle fed toxic or non-toxic fescue [13, 15] or between cattle fed high or low levels of toxic fescue [14]. Therefore, the physiological and genetic mechanisms associated with differences between type of fescue consumed by animals should be very different than those mechanisms associated with contrasting response to FT. Overall, our study suggests that there is a disruption in basic molecular and metabolic processes in these animals. Given that there was a much greater number of upregulated genes in LT animals compared to HT (Fig. 2B), we could postulate that this disruption is accentuated in LT animals, as a positive feedback to try to respond to the negative effects of FT. This greater gene expression pattern observed in LT animals is in accordance to other studies, such as between susceptible versus resistant Atlantic salmon to Infectious Pancreatic Necrosis virus [71] between cattle infected or not with Alcelaphine herpesvirus 1 [72] and between cattle experiencing or not severe pulmonary hypertension [73], as between cattle with and without dietary under stress [74].

Relationship with other phenotypes and limitations

Although we observed general biological processes associated with the DEG (Table 3) between HT and LT cattle, the most significant DEG (Table 2) had more specific and relevant functions for response to FT, such as for immune response and vasoconstriction activities. Hence, the classification of animals as HT or LT based on their growth during pregnancy seemed to have been appropriate. In fact, using the same animals as in this study, [26] reported that HT animals had greater body condition score, pregnancy rate by artificial insemination, and calf birth weight, and lower rectal temperature, hair shedding score, and hair coat score than LT animals at UPRS. In addition, this author also reported differences in cytokine concentrations between these two groups, such as G-protein coupled receptor-associated sorting protein 1, interleukin-4, and Vascular endothelial growth factor A. Furthermore, using the same set of animals, Koester et al. [75] identified contrasting fecal microbial communities between HT and LT animals. Altogether, these results suggest that the clear contrasting transcriptomic patterns between these two groups are associated with performance for several other phenotypes. In this study, we collected samples during late April to August from the blood of pregnant Angus cows. It would be interesting to evaluate genetic response to FT in spring calving cows and other tissues, such as in the rumen and liver, with the objective of evaluating digestive-related tissues.

Conclusions And Implications

In this study, we classified cattle into either highly tolerant or lowly tolerant to fescue toxicosis and used blood samples from these animals to identify genes with contrasting expression patterns between these

two groups. To our knowledge this study is the first attempt to identify the DEG associated with within variation for response to fescue toxicosis. Genotype-by-environmental interactions were evident in this study, as differences between tolerance groups were much greater at one location (UPRS), which had greater levels of toxic fescue than the other location (BBFCL). Moreover, samples were taken across time, and week 5 of the trial as the time-point in which differences in gene expression between tolerance groups were the greatest. The most significantly differentially expressed genes between tolerance groups have very relevant functions associated with fescue toxicosis: cellular growth (*SPDYC, HEYL, ANXA13*), cardiovascular function (*FGB*, *GLNC1*, *WNT11*, *BPIFB4*, *MESP2*), protein metabolism (*ENPP6, MMP8*), and immune response (*CTBS*, *SDC2*, *CXCL13*, *IL-13*, *JAKMIP2*). In addition, when all differentially expressed genes were analyzed, the enriched biological processes associated with fescue toxicosis included immune response, blood and cardiovascular function and development, cellular and biological processes, and fertilization.

These findings provide potential genomic biomarkers that should be evaluated for selection of cattle with greater tolerance to fescue toxicosis. In other words, genes identified in this study could be used to identify cattle that are tolerant to fescue toxicosis. This would help to establishing herds with fescue tolerant cows in regions in which high endophyte infection in fescue pastures is observed. Finally, additional studies should perform in order to better understand this devastating disease. For example, studies could focus on the identification of SNPs in these candidate genes, the use of other tissues to identify differentially expressed genes, identification of genomic regions associated with fescue toxicosis response, evaluation of different physiological states, and more.

List Of Abbreviations

FT: Fescue toxicosis

DEG: Differentially expressed genes

HT: High tolerant

LT: Low tolerant

SPDYC: Speedy/RINGO cell cycle regulator family member C

HEYL: Hes related family bHLH transcription factor with YRPW motif-like

ANXA13: Annexin A13

FGB: Fibrinogen beta chain

HBA: Hemoglobin subunit alpha

WNT11: Wnt family member 11

BPIFB4: BPI fold containing family B member

MESP2: Mesoderm posterior bHLH transcription factor 2

ENPP6: Ectonucleotide pyrophosphate/phosphodiesterase 6

MMP8: Matrix metallopeptidase 8

CTBS: Chitiobiase

SDC2: Syndecan 2

CXCL13: C-X-C motif chemokine ligand 13

IL-13: Interleukin 13

JAKMIP2: Janus kinase and microtubule interacting protein 2

SNP: Single nucleotide polymorphism

XKR4: Kell blood group complex subunit-related family, member 4

DRD2: Dopamine receptor D2

BBCFL: Butner cattle field laboratory

UPRS: Upper piedmont research station

AWG: Average weekly gain

AWG_res: Average weekly gain residual

GO: Gene Ontology

VARS2: Valyl-tRNA synthetase 2

LRRC47: Leucine rich repeat containing 47

RARS2: Arginyl-tRNA synthetase 2

DARS: Aspartyl-tRNA synthetase

FARSA: Phenyl alanyl-tRNA synthetase subunit alpha

EXOSC9: Exosome component 9

PAN2: Poly (A) specific ribonuclease subunit

LARS2: Leucyl-tRNA synthetase 2

Declarations

Ethics approval

All animal procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee (protocol #13-093-A).

Consent for publication

Not applicable

Availability of data materials

The data are available from the corresponding author on reasonable request.

Competing interest

The authors declare there are no competing interests.

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Author Contributions

PK analyzed the data, interpreted the results and drafted the manuscript; LPS helped in data analysis and interpreting the result; KM was involved in collection of sample and interpretation of result; JS lead RNA extraction and sequencing, and interpretation of results, MHP was involved in designing experiment, interpretation of results and paper revision, DHP was involved in designing the experiment, collecting the sample, interpretation of results and paper revision, NVLS was involved in designing the experiment, collecting sample, interpreting the results and providing consultation for all the analyses. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, all Tables are only available as a download in the supplemental files section.

Figures

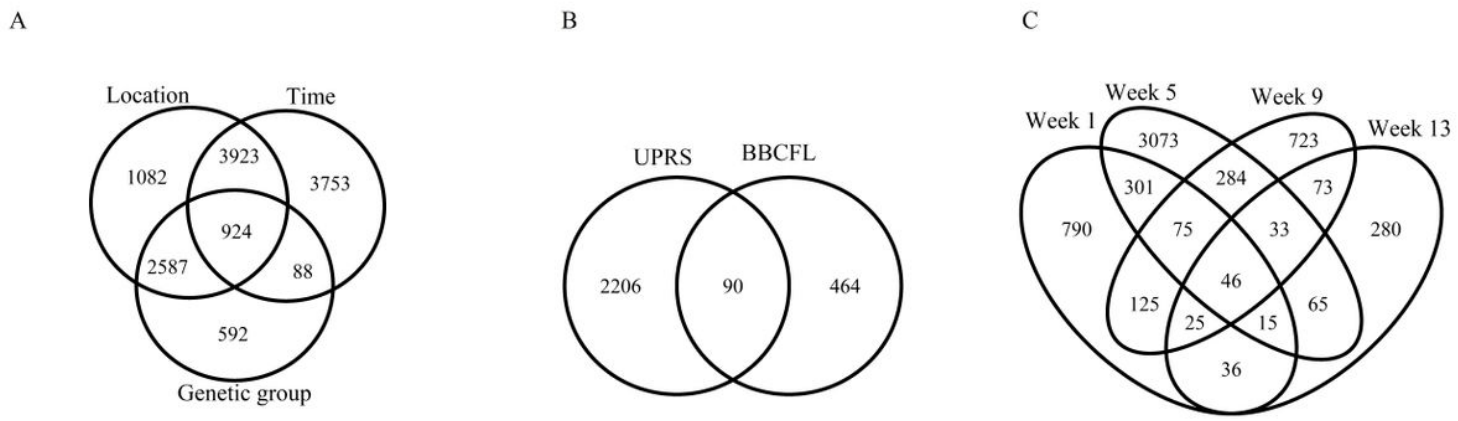

Figure 1

Venn diagram with the number of differentially expressed genes (DEG; p-value < 0.1). (A) Venn diagram for the effects of genetic group, location, time, genetic group-by-time, genetic group-by-location, time-by-location and genetic-group-by-location-by-time; where the numbers represents the uniquely identified DEG, respecting the hierarchy of higher order interactions; (B) Venn diagram for the effect of genetic group within each location: Upper Piedmont Reidsville Station (UPRS) and Butner Beef Cattle Field Laboratory (BBCFL); and (C) Venn diagram for the effects of genetic group across weeks.

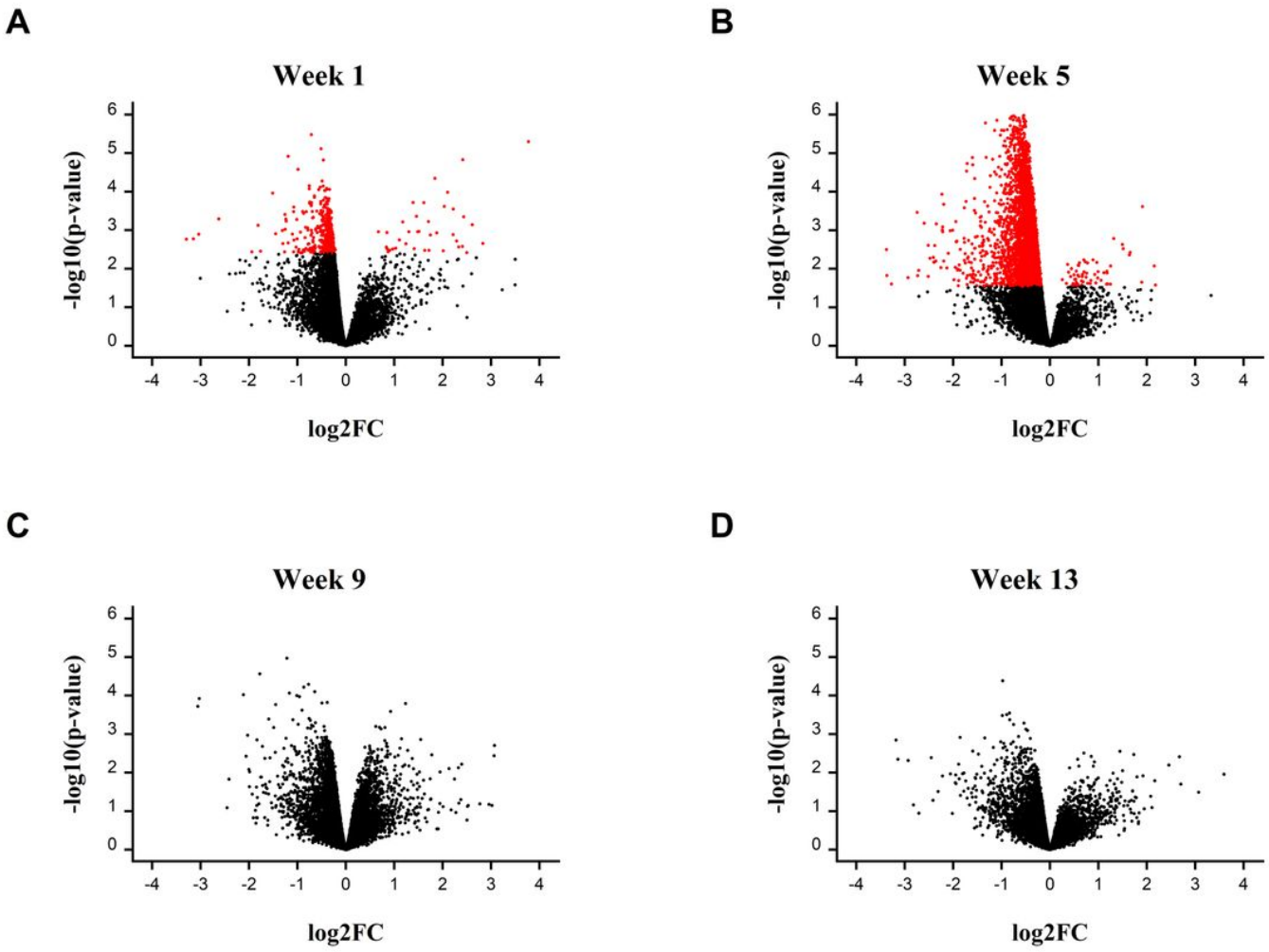

Figure 2

Volcano plots for the effects of genetic group at Upper Piedmont Reidsville Station (UPRS) on weeks 1 (A), 5 (B), 9 (C), and 13 (D). X- axis represents the log2 fold change (log2FC) between the two genetic groups. Negative and positive log2FC values represent gene upregulation in low tolerance (LT) and high tolerance (HT) animals, respectively. Y-axis represents the negative log10 P-value. Differentially expressed genes (q-value < 0.1) between genetic groups are highlighted in red.

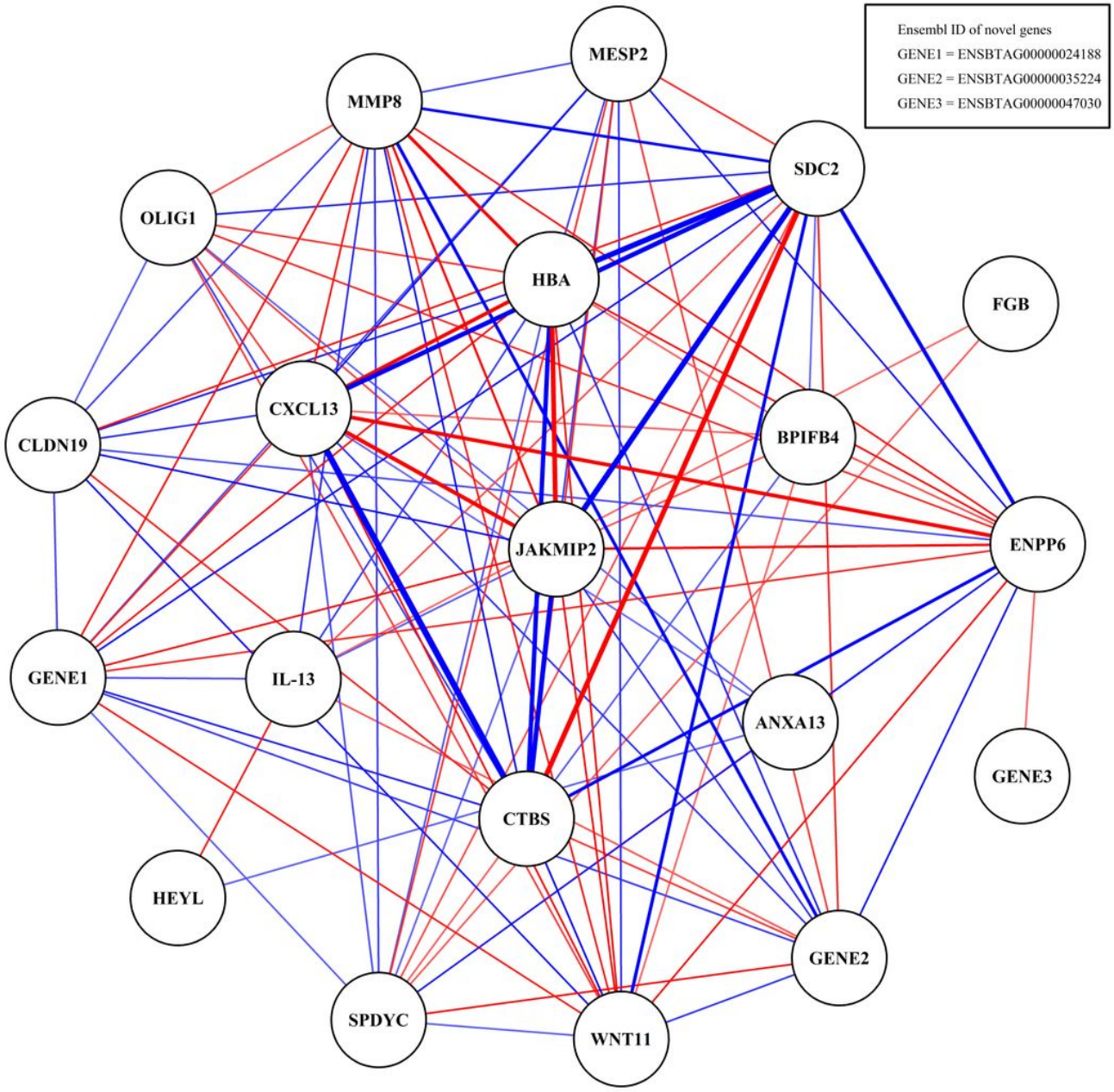

Figure 3

Partial correlation network for the top 20 differentially expressed genes (Table 2) for the effect of genetic group at Upper Piedmont Reidsville Station (UPRS) at week 5. The blue and red edges represent positive and negative correlations, respectively. The thickness of the edge represents the strength of the correlation with wider edges, stronger the partial correlation. A threshold of partial correlation of |0.5| was used to reduce the number of connections.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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