Metabolomics of fescue toxicosis in grazing beef steers

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List of Abbreviations:

12-HETE, 12-hydroxyeicosatetraenoic acid; 5HT, serotonin; 5HT1B, serotonin 1B receptor; 5HT1D, serotonin 1D receptor; 5HT2A, serotonin 2A receptor; AA-5-HT, arachidonyl serotonin; AE, anion-exchange liquid chromatography; AFMK, N(1)-acetyl-N(2)-formyl-5-methoxykynuramine; apLCMS, adaptive processing liquid chromatography mass spectrometry; BW, body weight; C18, reverse phase liquid chromatography; D2, dopamine-2 receptor; DA dopamine; DHBA, 3,4-dihydroxybenzylamine; DOPAC, 3,4-dihydroxyphenyl acetic acid; E, epinephrine; ELISA, enzyme linked immunosorbent assay; EPI, epinine; FAAH, fatty acid amide hydrolase; FT, fescue toxicosis; FT-ICR, Fourier transform ion cyclotron resonance; HMDB, Human Metabolome Database; HPLC-ECD, high performance liquid chromatography with electrochemical detection; HRM, high-resolution metabolomics; HVA, homovanillic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC, liquid chromatography; L-DOPA, 3,4-dihydroxyphenylalanine; Lipid MAPS, Lipid Metabolites and Pathways Strategy database; LTQ, linear triple quadrupole; m/z, mass to charge ratio; Metlin, Scripps Center for Metabolomics Metlin batch processing software; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NE, norepinephrine; NT, non-toxic; PAF, platelet activating factor; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; T, temperature; THI, temperature humidity index; VFA, volatile fatty acid.

Abstract

Fescue toxicosis (FT) results from consumption of tall fescue (*Lolium arundinaceum*) infected with an endophyte (*Epichloë coenophiala*) that produces ergot alkaloids (EA), which are considered key etiological agents of FT. Decreased weight gains, hormonal imbalance, circulating cholesterol disruption, and decreased volatile fatty acid absorption suggest toxic (E+) fescue-induced metabolic perturbations. Employing untargeted high-resolution metabolomics (HRM) to analyze E+ grazing-induced plasma and urine metabolome changes, fescue-naïve Angus steers were placed on E+ or non-toxic (Max-Q) fescue pastures and plasma and urine were sampled before, 1, 2, 14, and 28 days after pasture assignment. Plasma and urine catecholamines and urinary EA concentrations were also measured. In E+ steers, urinary EA appeared early and peaked at 14 days. 13,090 urinary and 20,908 plasma HRM features were detected; the most significant effects were observed earlier (2 days) in the urine and later (>14 days) in the plasma. Alongside EA metabolite detection, tryptophan and lipid metabolism disruption were among the main consequences of E+ consumption. The E+ grazing-associated metabolic pathways and signatures described herein may accelerate development of novel early FT detection and treatment strategies.

Keywords: fescue toxicosis, *Epichloë coenophiala*, ergot alkaloids, grazing beef cattle, untargeted metabolomics

1. Introduction

Tall fescue, *Lolium arundinaceum*, is a valuable forage grass that is frequently infected with the endophytic fungus *Epichloë coenophiala*, previously known as *Neotyphodium coenophialum* (Young et al., 2014). The relationship between the plant and endophyte is viewed as mutualistic as the fungus produces secondary metabolites that protect against extrinsic plant fitness factors and increase stand persistence (Clay, 1990b; Clay, 1993; Hill et al., 1990; Read and Camp, 1986). Thus, endophyte-infected tall fescue is a sustainable grass forage option that outcompetes other plants exposed to similar environmental conditions (Clay, 1990a, b). Today, endophyte-infected tall fescue covers approximately 14 million hectares of pasture land across the Southeastern United States (Young et al., 2014).

Despite the benefits of this mutualistic relationship, the wild-type endophyte is referred to as toxic (E+) due to its production of toxic-to-the-animal ergot alkaloids (EA) and consumption of E+ fescue causes the development of fescue toxicosis (FT), a disease with an estimated one-billion-dollar detriment to the United States beef industry (Craig et al., 2015; Matthews and Haley, 2015; Strickland et al., 2011). Drivers of these economic losses include lowered weight gains and reduced fertility (Bacon, 1995; Craig et al., 2015; Edite Bezerra da Rocha et al., 2014; Thompson and Stuedemann, 1993). The main EA produced by the toxic endophyte is ergovaline (comprising 80-100% of the ergopeptide fraction); however, other toxic EA are also produced by the E+ endophyte (Gadberry et al., 2003; Guerre, 2015).

Non-toxic endophyte-containing fescue varieties are available (Hancock and Andrae, 2012; Hopkins and Alison, 2006; Klotz et al., 2013), but replacement of E+ endophyte-infected tall fescue is not a popular option for remediation (Clay, 1990b; Young et al., 2014). Non-toxic endophytes do not produce EA; the stand persistence and yield benefits are provided from the production of, among others, peramine, terpendole, and N-acetylnorloline without the decreased steer performance seen when grazing E+ fescue (Bouton et al., 2001; Young et al., 2014; Young et al., 2013). Despite this, switching to novel non-toxic endophytes requires a time lag before the land becomes profitable again, can cause

soil erosion, and impairs pasture sustainability (Bouton et al., 2002; Guerre, 2015; Gunter and Beck, 2004; Hancock and Andrae, 2012; Hoveland, 1993; Parish et al., 2003; Pimentel and Burgess, 2013).

Several contributing mechanisms to FT have been identified. Vasoconstrictive responses (Klotz et al., 2008; Oliver et al., 1993; Oliver et al., 1998; Rhodes et al., 1991) inducing perturbed nutrient absorption and metabolism (Egert et al., 2014a; Pesqueira et al., 2014) occur concurrently with altered circulating prolactin and cholesterol levels, which together with and intra- and extrahepatic enzyme activity changes, may play a role in the etiology of FT (Bacon, 1995; Rosenkrans and Ezell, 2015; Settivari et al., 2008; Thompson and Stuedemann, 1993; Zbib et al., 2014, 2015). Foote et al. (2013) suggested impaired weight gain might be attributed to a decrease in volatile fatty acid (VFA) absorption or production caused by increased ruminal EA concentration. In ruminants, VFAs are a major energy source (Dijkstra et al., 1993), and decreased VFA absorption could lead to a decreased metabolic rate and, ultimately, decreased food intake and weight gains if chronic exposure occurs (Foote et al., 2014).

Detection of EA in the urine has been shown as an accurate, less variable ergot alkaloid exposure diagnostic tool when compared to previous methods (Hill et al., 2000; Stuedemann et al., 1998); ninety-four percent of EA excretion is urinary, with EA appearing as early as 12 hours post-exposure and concentrations being exposure level- and duration-dependent (Hill et al., 2000). Speciating ergot alkaloids and metabolites, in plasma and urine, might help identify how ruminants metabolize EA, and how other metabolic processes may be affected (Hill et al., 2000; Hill et al., 2001).

Ergot alkaloids share structural similarity with dopamine (DA), norepinephrine (NE), and serotonin (5-HT) (Klotz and Nicol, 2016) and exhibit dopaminergic, adrenergic, and serotonergic activities (Berde and Stürmer, 1978; Müller-Schweinitzer and Weidmann, 1978; Pertz and Eich, 1999) that influence numerous physiological functions (Dyer, 1993; Dyer, 2000; Klotz, 2015; Larson et al., 1995; Larson et al., 1994; Oliver, 1997, 2005; Oliver and Abney, 1989; Oliver et al., 1993; Vanhoutte and Luscher, 1986). Thus, EA can potentially decrease appetite and food intake through modulation of the hypothalamic serotonergic tone (Rossi-Fanelli and Cangiano, 1991). Sheep plasma DA levels

increase (Henson et al., 1987) and plasma 3,4-dihydroxyphenyl acetic acid (DOPAC; a dopamine metabolite) levels in mares decrease (Youngblood et al., 2004) in response to toxic fescue consumption. Plasma levels of 5-HT, NE, homovanillic acid (HVA; another DA metabolite), and epinephrine (E) in response to E+ consumption are not well characterized for any agricultural species.

Metabolomics can be used to determine how toxicants affect crucial metabolic pathways and mechanisms of disease pathogenesis, potentially leading to novel exposure or effect biomarker identification (Li et al., 2013; Li et al., 2014; Lin et al., 2014). In dairy cattle, metabolomics has been used to profile metabolic signatures associated with milk fever (Sun et al., 2014), clinical ketosis (Li et al., 2014), and periparturient diseases (Hailemariam et al., 2014). In beef cattle, disease/condition-specific metabolomics-based approaches have not been utilized thus far.

High-resolution metabolomics (HRM) uses liquid chromatography (LC) coupled to ultra-high resolution mass spectrometry (MS) and advanced data extraction methods to detect thousands of metabolic features quickly (Walker et al., 2016). Samples are analyzed in triplicate to enhance the reliability of detection of low abundance chemicals, and data extraction methods utilize adaptive processing LCMS (apLCMS; Yu et al., 2009) and automated re-extraction, statistical filtering and data merger to enhance data quality (Uppal et al., 2013). To overcome a bottleneck metabolite identification in this information-rich data, statistical tests and bioinformatics methods are used to select mass spectral features associated with endpoints of interest (e.g., consequences of EA exposure), and computer algorithms are then used to test for pathway enrichment (Dunn et al., 2012; Li et al., 2013).

In the present study, HRM was utilized to analyze the plasma and urine metabolomes of beef cattle after grazing E+ or non-toxic endophyte-infected tall fescue to test for metabolic differences associated with ergot alkaloid exposure. Total urinary EA and targeted analyses of select catecholamines and their metabolites were also measured. Post HRM processing, metabolite annotation, network analysis, and pathway analysis were conducted using reference metabolite databases and bioinformatics

processing (Deo et al., 2010; Li et al., 2013; Ma et al., 2007) for an in-depth look into the metabolic pathways that are perturbed after E+ grazing.

2. Materials and Methods

2.1. Animals and Treatments

The study was conducted in the fall of 2013 (Sept. 16 – Oct. 14). All sample collection was completed in the morning, between 8-11AM. The pastures were located at the J. Phil Campbell Natural Resources Conservation Center of the University of Georgia (Watkinsville, GA), and temperature (T) and relative humidity measurements were collected using an on-site weather station. Fescue-naïve Angus steers (n=8; weighing 199.5 ± 10.5 kg (mean $\pm$ SEM) and 200.6 ± 10.5 kg for toxic (E+) and non-toxic (Max-Q) groups, respectively; 4 steers per fescue treatment; 2 paddocks per treatment with 2 steers per fescue paddock) were randomized and assigned to fall-saved 0.8 hectare vegetative fescue pastures that were sown in the fall of 2004 with either a novel non-toxic endophyte (Jesup MaxQ strain AR542 containing less than 1 ppb ergot alkaloid; Max-Q) that does not decrease animal performance (Hancock and Andrae, 2012; Hopkins and Alison, 2006) or a toxic endophyte (Jesup with wild-type endophyte; E+). The steers were maintained on their respective pastures for 28 days. At baseline (time 0), 1, 2, 14, and 28 days post pasture assignment, plasma and urine were collected and processed for HRM (Li et al., 2013; Patel et al., 2015), urinary ergot alkaloid analysis (Hill et al., 2000), and plasma and urine catecholamine analysis (Smith et al., 2013; Youngblood et al., 2004). Additional details of these analyses are provided below.

Determining the effects of E+ fescue on weight gain was not an objective of this study. Nevertheless, the steers were weighed prior to treatment assignment and then again approximately a month after completion of the study prior to their sale; at both times, the two groups' weights were not different. Thus, the mean steer weight prior to the study (provided above) was not different ($P > 0.8$) between the E+ and Max-Q steers. Similarly, steers' BW one month after termination of the study (279.6 [Max-Q] and 283.1 [E+] kg, respectively) was not different between groups ($P > 0.9$).

All animal handling and sample collection methods were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

2.2. Sample Collection and Processing

Blood samples were taken from the jugular vein and placed in K₃ EDTA vacutainer tubes (Becton Dickinson and CO, Franklin Lakes, NJ) on ice until plasma was harvested. Voided urine samples were taken by urine collection in sterile cups and placement on ice in 15-mL conical centrifuge tubes (Fisher Scientific, Waltham, MA). Upon arrival to the lab, urine samples were immediately centrifuged (300 x *g* for 10 min at 4 °C), aliquoted and stored at -80 °C until further analysis. Plasma was collected after centrifuging the blood (3,500 x *g* for 30 min at 4 °C) and stored at -80 °C until further analysis.

2.3. Metabolomics Sample Processing and Data Analysis

2.3.1. Metabolomics Sample Processing

Plasma and urine samples (1 mL aliquots) were processed as previously described for human plasma and urine (Patel et al., 2015). Briefly, randomized samples were treated with 2 volumes of ice-cold acetonitrile containing an internal standard of a mixture of 14 stable isotopes, incubated on ice for 30 min, and centrifuged (14,050 x *g* for 10 min) to remove any precipitated proteins. The isotopes used for the internal standard were: [¹³C₆]-D-glucose, [¹⁵N]-indole, [2-¹⁵N]-L-lysine dihydrochloride, [¹³C₅]-L-glutamic acid, [¹³C₇]-benzoic acid, [3,4-¹³C₂]-cholesterol, [¹⁵N]-L-tyrosine, [trimethyl-¹³C₃]-caffeine, [¹⁵N₂]-uracil, [3,3-¹³C₂]-cystine, [1,2-¹³C₂]-palmitic acid, [¹⁵N,¹³C₅]-L-methionine, [¹⁵N]-choline chloride, and 2'-deoxyguanosine-¹⁵N₂,¹³C₁₀-5'-monophosphate. Samples were kept in a refrigerated autosampler prior to a 10-μL injection. Urine samples were analyzed using a Thermo Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Thermo Fisher, San Diego, CA) set to collect data from a mass to charge ratio (*m/z*) 85 to 850; plasma samples were analyzed using a Thermo linear triple quadrupole (LTQ) Velos Orbitrap mass spectrometer (Thermo Fisher) set to collect data from *m/z* 85 to 2000. Both mass spectrometers were coupled with either anion-exchange (AE) or reverse phase (C18) liquid chromatography (LC). All samples were run in triplicates utilizing both forms of LC, with a

pooled reference sample, which was calibrated to the NIST SRM 1950, run every 21st sample (Phinney et al., 2013). The chromatography was performed with a temporal acetonitrile gradient for 10 min. A flow rate of 0.35 ml/min was used for the first 6 min and 0.5 ml/min for the remaining 4 min. The first 2-min period consisted of 5% A (water with 2% formic acid), 60% water, 35% acetonitrile, followed by a 4-min linear gradient to 5% A, 0% water, 95% acetonitrile. The final 4-min period was maintained at 5% A, 95% acetonitrile, with flow rate set at 0.35 mL/min for the first six min before changing to 0.50 mL/min for the final 4 min. Data were stored as raw spectral files and converted to computable document format files using Xcalibur file converter software (Thermo Fisher). Peak detection, noise filtering, m/z and retention time alignment, feature quantification, and quality filter were performed using xMSanalyzer v2.0.7 (Uppal et al., 2013) with apLCMS v6.1.3 (Yu et al., 2009). Data were extracted as m/z features, defined by m/z , retention time, and integrated ion intensities. The average of the three technical replicates was used for subsequent bioinformatics analysis. Features that had at least 70% signal regardless of experimental condition were used to identify differentially expressed metabolites between treatment groups and collection times (Patel et al., 2015).

2.3.2. Plasma and Urine Metabolite Annotation

After averaging the integrated ion intensities for each m/z feature for each animal, statistically significant differences were determined with a two-sided t-test ($P < 0.05$). Venn Diagrams were generated using the Bioinformatics and Evolutionary Genomics online processor (<http://bioinformatics.psb.ugent.be/webtools/Venn/>), by entering the m/z ratios for the significantly ($P < 0.05$) different metabolites into the processor. Annotation was completed using the Scripps Center for Metabolomics Metlin batch processing software (Metlin; <https://metlin.scripps.edu/index.php>) by taking the significantly different m/z features and querying the Metlin batch processor with a conservative $\Delta 5$ ppm tolerance.

2.3.3. Metabolomics Data Processing

Using *R* language version 3.2.2. (R Core Team, 2015), principal component analysis (PCA) and

partial least squares discriminant analysis (PLS-DA) were completed using the *mixOmics R* package (Le Cao et al., 2015), with the *Reshape* package (Wickham, 2007) for data frame shaping; feature peak intensities from each LC column were used as analysis variables for both biological matrices across all time points and within a specific time point post pasture assignment. Volcano plots were generated using metabolomics feature peak intensities in the *metabolomics R* package (De Livera and Bowne, 2014) for each time point, biological matrix, and column; the metabolites that were significantly different ($P < 0.05$) were extracted and analyzed as described in Section 2.3.2. Heat maps were generated using the *gplots* library (Warnes et al., 2015), with peak intensities of significantly ($P < 0.05$) different metabolites used for the analysis.

2.3.4. Network and Pathway Analysis

Network analysis was completed using the *MetabNet R* package (Uppal, 2015) coupled with Cytoscape v3.2.1 network visualization software (Shannon et al., 2003), where a complete Pearson correlation-based network analysis was performed for each biological matrix, with correlation stringencies ($|r|$) set at ($|r| > 0.5$) and false discovery rate (FDR) at $FDR = 0.05$. The target list of metabolites used for network analysis was rich with tryptophan, tyrosine, and ergot alkaloid metabolites.

Pathway analysis was completed by taking the putatively identified metabolites of interest and querying the following databases: Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>), Metlin, the Human Metabolome Database (HMDB; <http://www.hmdb.ca>), and the Lipid Metabolites and Pathways Strategy database (Lipid MAPS; <http://www.lipidmaps.org>). From this information, pathways that were differentially regulated, i.e., multiple significantly different metabolites within the same pathway in the plasma and/or urine metabolomes, were constructed.

2.4. Total Plant and Urinary Ergot Alkaloids Analysis

Individual tillers were sampled from pastures on 28 September, 2013. Sampling was performed by selecting a tiller from each of 100 locations within the pastures, cutting the tiller at the soil surface, and

transporting the samples to the laboratory. Endophyte presence was analyzed from a 3-mm cross section of the stem base of each tiller using a commercial immunoblot test kit (Agrinostics Ltd. Co., Watkinsville, GA, Cat. # ENDO797-3). In addition, plant ergot alkaloid presence or absence was determined by a second tiller cross section using a commercial ELISA test kit (Agrinostics Ltd., Cat. # ENDO899-2t). The remaining plant tissue was frozen, freeze-dried, ground to pass a 1-mm screen, and analyzed for total ergot alkaloid using a commercial ELISA kit (Agrinostics Ltd., Cat. # END0899-96p). The detection limits for the two alkaloid test kits are 0-25 and 0-2000 $\mu\text{g}/\text{kg}$, respectively. Mean endophyte presence in the E+ pastures was 95% and all plant tillers with endophyte tested positive for EA. Mean endophyte presence in the Max-Q pastures was 92% with no EA present in any tillers. The ergot alkaloid concentration in the whole tissue samples was 1024 and 0 $\mu\text{g}/\text{kg}$ for the E+ and Max-Q pastures, respectively.

Total urinary EA concentrations were determined at 1, 2, 14, and 28 days post-pasture assignment ELISA (Agrinostics Ltd.) as previously described (Hill et al., 2000; Hill and Agee, 1994; Hill et al., 1994). Urine samples were serially diluted (1:2 to 1:16) before analysis and lysergic acid was used as the standard. The urine was also analyzed for creatinine to normalize the urinary EA prior to statistical analysis (Murray, 1989); values are presented as ng of ergot alkaloid per mg of creatinine.

2.5. Plasma and Urine Catecholamine Sample Processing and Analysis

2.5.1. Sample Processing

Sample processing for catecholamine extraction was adapted from (Youngblood et al., 2004) for plasma and (Smith et al., 2013) for urine. Briefly, for plasma, 50 mg acid activated alumina, 500 μL sample, 500 μL 0.2 M Tris buffer (pH 9.0), and 20 μL of internal standard (epinine, 2 ng) were incubated for 15 min at room temperature on a rocker. The samples were centrifuged at 500 x g for one min at 23 °C, supernatant was removed, and 1 mL Na_2EDTA (1.1g/L) was added to wash the sample. The sample was vortexed, and washed twice more (3 washes total). The supernatant was then removed, 200 μL 0.2 N HClO_4 was added, the sample was vortexed for 15 sec, spun as before, and final

supernatant was collected and stored at -80°C until analysis. For the urine (Smith et al., 2013), 50 mg acid activated alumina, 1 mL 0.05 N HCl with 5 mM $\text{Na}_2\text{S}_2\text{O}_5$, 100 μL urine, 20 μL internal standard, and 1 mL 0.2 M Tris (pH 9.0) were mixed, and the pH was adjusted to 8.5. The mixture was vortexed and incubated at room temperature for 5 min on a rocker. The samples were centrifuged as the plasma samples, the supernatant was removed, 1 mL 18.2 m Ω water was added, and the sample was vortexed and centrifuged as before; this was repeated one more time. Then, supernatant was removed, 200 μL of 0.1 N HClO_4 with 0.1 mM $\text{Na}_2\text{S}_2\text{O}_5$ was added, the sample was vortexed for 15 sec, and spun as before. For both plasma and urine, the processed samples were transferred to a microcentrifuge tube, spun at 13,200 $\times g$ for 3 min at 4°C , and the supernatant was then either set up for injection into the HPLC or stored at -80°C until analysis.

2.5.2. HPLC-ECD and Data Analysis

A mixed catecholamine standard containing 2 ng (20 μL) each of dopamine (DA), L-3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), epinephrine (E), homovanillic acid (HVA), and two internal standards consisting of 3,4-dihydroxybenzylamine (DHBA) and epinine (EPI), was prepared in with 0.2 N HClO_4 and injected onto the column (SUPELCOSIL[™] LC-18-DB HPLC Column 5 μm particle size, $L \times \text{I.D.}$ 25 cm $\times$ 10 mm; Supelco Inc., Bellefonte, PA). The mobile phase (pH 3.65) consisted of monobasic sodium phosphate, octyl sodium sulfate, EDTA, methanol, H_2O , and trimethylamine (Youngblood et al., 2004).

A Waters e2695 HPLC using Empower Pro software and a Waters 2465 electrochemical detector (ECD) was used for all analysis, as in Youngblood et al. (2004), with the flow rate set at 1 mL/min and the detector set at 850 mV. Analyte peaks in standards and samples were integrated and quantitated based on epinine recovery (plasma) and epinine recovery and creatinine normalization (urine).

2.6. Statistical Analysis of Non-Metabolomics Data

The urinary alkaloids and plasma/urine catecholamines were analyzed by Sigma Plot v12.5 (Systat Software Inc., San Jose, CA). Urinary alkaloids, DA, NE, E, DOPAC, and HVA values were analyzed

by two-way ANOVA, with period and fescue treatment set as the two independent variables. If significant ($P < 0.05$) main effects or interactions were present, Student-Newman-Kuels *post-hoc* was used to separate significant differences.

3. Results

3.1. Environmental Conditions

For the duration of the study, the average daily T was 19.5 °C (range: 16.6 -23.6) and the average temperature-humidity index (THI; Kibler, 1964) was 66.2 (range: 62.1-72.7); these two averages for the five sampling dates were, respectively, 20.4 °C and 67.4. Except for day 0, when the T and THI were 23.6 and 72.7, respectively, steers were not subjected to environmental conditions associated with even mild heat stress (i.e. the THI was ≤ 72 ; Armstrong, 1994; Supplemental Figure 1).

3.2. Ergot Alkaloids: Quantitation by ELISA and Detection using HRM

Total urinary EA in E+ steers increased markedly ($P < 0.05$) from day one to day two and, again, from day two to day 14 before leveling off at 28 days (Figure 1). Urinary EA in Max-Q steers were very low and undetectable in most cases (range: 0.00 – 6.26 ng/mg creatinine).

Via HRM, we were able to identify multiple putative EA in both plasma and urine. In the E+ plasma, the number of putative EA identified having a basic ergoline ring structure was consistent throughout the grazing duration, except after 2 days of grazing where there was a decrease (Table 3A). Total number of putatively identified EA in the urine peaked 2 days after exposure, was stable after 14 days, and decreased after 28 days (Table 3B). The majority of the putative urinary EA were ergoline derivatives/lysergic acid amides, with two ergopeptides appearing after 14 days of E+ grazing (Table 4). The number of putative ergopeptides in the plasma increased with time spent on pasture, with a total of 5 unique ergopeptides identified after 14 and 28 days of grazing (Table 4).

3.3. Plasma and Urine Catecholamines

As a whole, plasma and urine catecholamines were variable. No significant treatment differences were observed for any plasma catecholamines, i.e., they were not affected by E+ grazing to a major extent or in a consistent direction; there were some numerical trends, i.e., plasma DA ($P = 0.16$; data not

shown) and E ($P = 0.14$; data not shown) levels were numerically higher in E+ steers after 28 days of grazing. Urinary DA, E, and NE levels fluctuated significantly in both treatment groups, but the timing of the fluctuation differed. As a result, DA, E, and NE in the urine were lower in E+ steers after 2 days of grazing ($P < 0.05$; Figure 2A, 2B and 2C); urinary DA and E significantly ($P < 0.05$) and NE numerically ($P = 0.11$) increased in E+ steers after 28 days of grazing (Figure 2A and 2B). There was also an overall decrease in urinary DOPAC levels ($P < 0.05$), which was driven by decreases in E+ steers at 1, 2, and 14 days (Figure 2D and 2D inset).

3.4. Metabolomics of E+ Fescue Grazing: Feature Collection Results

After HRM processing, a total of 6,478 and 6,612 unique features (i.e., compound with unique m/z and retention time) were detected in the urine on the AE column and C18 columns, respectively (Table 1). Of these, 457 (AE) and 634 (C18) features were significantly different between the treatment groups (Table 2; $P < 0.05$). In the plasma, a total of 9,403 (AE) and 11,505 (C18) unique features were detected (Table 1), with 1,179 (AE) and 1,415 (C18) of them being significantly ($P < 0.05$) different between the two groups (Table 2). The largest number of significantly different metabolites in the urine occurred after 2 days of grazing; in the plasma, the largest number of significantly different features was seen at 14 and 28 days (Table 2). Some significantly different m/z features overlapped between different time points and Venn Diagrams were generated to evaluate significant treatment differences over the course of the study (Figure 3). The greatest overlap was seen between 14- and 28-day plasma samples (36 total) and between 1- and 2-day urine samples (23 total; Figure 3). Also, several m/z features overlapped across all four time points (plasma: 5, urine: 11; Figure 3).

3.5. Further In-Depth Analysis of Metabolomics Data

Volcano plots, PLS-DA, and heat maps are common statistical analysis methods used to analyze statistical variations and trends in metabolomics studies. Volcano plots were generated for visualization of metabolites that were significantly different between treatments. As illustrated in Figure 4A (AE) and 4B (C18) for the 28-day plasma and in Figure 4C (AE) and 4D (C18) for the 2-day urine, there were

multiple significantly different metabolites with large fold changes ($P < 0.05$; fold change ± 2), but there were also metabolites that were highly significantly different ($P \leq 0.01$) with smaller fold differences (fold change < 2). All significantly different metabolites ($P < 0.05$) from all time points, irrespective of fold change, were extracted and taken through the metabolite annotation step (Section 2.3.2).

From PLS-DA, steers grazing Max-Q and E+ tall fescue formed distinct clusters (C18 data: Figure 5; AE data: not shown). When PLS-DA was performed including all time points for plasma (C18 data; Figure 5A) and urine (C18 data; Figure 5C), the Max-Q and E+ treatments separated into two distinct clusters of non-overlapping nature. Similar separation was observed for the AE column (data not shown). Much like the combined data, there was marked treatment separation in the PLS-DA plots for 28-day plasma (Figure 5B) and 2-day urine (Figure 5D) C18 data; similar results were found within every sampling date and for AE column data (data not shown). Overall, these data point to treatment-related changes in the plasma and urine metabolomes of steers that appear early and persist.

After the PLS-DA analysis, heat maps were generated to analyze further the differences in the metabolomes between the E+ and Max-Q treatment groups and to determine if there is any indication of pasture (paddock) effect within treatment. The 28-day plasma AE and 2-day urine AE heat maps for significantly different m/z features (Figure 6A and 6B, respectively) are presented as examples, but heat maps for other time points/columns are similar. Overall, the heat maps for significantly different ($P < 0.05$) m/z features demonstrate clear metabolome differences between the Max-Q and E+ steers. On the other hand, the impact of pasture within grazing treatment was minimal (i.e., differences in color intensity on the heat maps between the two pastures within a treatment; Figure 6A and 6B).

3.6. Putative Metabolites of Interest

Initially focusing on significantly different putative metabolites that overlapped between 1 and 2 days, between 14 and 28 days, and among all time points in the two sample matrices, tyrosine and tryptophan metabolites (Table 5) were identified as two major metabolite groups affected by E+ grazing.

In the urine, significant increases of certain eicosanoids, i.e., 12-hydroxyeicosatetraenoic acid, were observed after 14 and 28 days of E+ grazing. In addition, urinary trace amines, i.e., phenylethanolamine and octopamine, were other putative metabolites that were significantly increased at each time point, with temporal increases occurring in E+ steers. Cortolone-3-glucuronide and tetrahydroaldosterone-3-glucuronide were increased after 1, 2, and 28 days of E+ grazing, whereas chlormadinone acetate, a progesterone antagonist, was increased in the urine at 2 days.

In the plasma, among the putative metabolites significantly impacted by E+ consumption were many glycerophospholipids, namely phosphatides and lecithins, along with several glycolipids, i.e., phosphatidylinositol, phosphatidylethanolamine, and 3-beta-D-galactosyl-sn-glycerol (Table 5). Most plasma glycerophospholipids increased by toxic fescue grazing, whereas some of the phosphatides decreased. Of note, arachidonyl serotonin (AA-5-HT), a lipid signaling molecule, increased in E+ steers at all sampling dates. Finally, metabolites involved in tryptophan and tyrosine metabolism were found to be significantly different (metabolite-specific increase or decrease) across all time points, but plasma and urine metabolite profiles were different (Table 5).

3.7. Network Analysis

Targeted networks were generated based on tryptophan, tyrosine, and ergot alkaloid metabolites that were previously identified as impacted by E+ grazing in Section 3.6. In the plasma (Figure 7), two clusters formed around associations between EA, serotonin, and indole metabolites, with the center of the main cluster being dihydroergocryptine, 5-methoxytryptamine, and indolylmethylthiohydroximate. Separate clusters, associated with methylergonovine and 5-hydroxyindoleacetyl glycine (Figure 7), indicate secondary metabolic processes are influenced based on the link between these two metabolites, the constituents of their clusters, and metabolites residing in the main cluster. Also, three separate clusters, apart from the main cluster, indicate associated effects on the anchors (tyramine,

dihydroergotamine, 5-hydroxyindoleacetyl-glycine) and constituents of these clusters without associations to the main cluster (Figure 7).

In the urine (Supplemental Figure 2), one main metabolite cluster centered around 5-hydroxy-6-methoxyindole and 5-hydroxyindoleacetaldehyde, with a slight cluster separation around tetrahydroaldosterone-3-glucuronide and cortolone-3-glucuronide. This indicates that tryptophan and steroid hormone metabolisms are affected in the urine, and several metabolites can be seen correlating with this perturbation. Finally, N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK), yellow node in Supplemental Figure 2, was multi-linked to main cluster metabolites, indicating a secondary metabolic process-type relationship.

3.8. Pathway Analysis

The tryptophan metabolic pathway was highly perturbed in E+ steers' plasma and urine, apparently impacted differentially (Figure 8). Four putatively identified metabolites in the plasma are directly involved in tryptophan metabolism, with a significant decrease of metabolism following the pathway from serotonin through melatonin end products (Figure 8A; i.e., 5-hydroxyindolacteyl-glycine), and a significant increase in metabolism of serotonin to 5-methoxytryptamine. The indole pathway of tryptophan metabolism was also effected, with significantly increased levels of tryptamine and indole-3-thiohydroximate, indicating increased tryptophan metabolism into tryptamine and its derivatives (Figure 8A). Tryptophan biosynthesis from the shikimate pathway had one increased putative metabolite, 3-dehydroshikimate, a precursor to anthranilate, which is an important precursor in the biosynthesis of both tryptophan and tyrosine (Figure 8A).

In the urine (Figure 8B), decreased levels of serotonin breakdown products and increased levels of the melatonin metabolite AFMK in E+ steers point toward increased metabolism of melatonin (Figure 8B).

Finally, tyrosine metabolism was affected in E+ steers across all time points, but the direction of the effects was inconsistent; this applies to both the plasma and urine (data not shown).

4. Discussion

Untargeted metabolic profiling of beef cattle plasma and urine is novel. Utilizing HRM and using two different columns allowed for better metabolite separation; we detected and quantified on average 6,500 and 10,500 unique features per column (AE and C18) in urine and plasma, respectively. The number of detected features in the plasma here is slightly lower than reported data for human plasma analyzed similarly (Burgess et al., 2015; Frediani et al., 2014; Patel et al., 2015). However, compared with other untargeted metabolomics applied to both plasma and urine in rodents or humans (Chen et al., 2012; Feng et al., 2016; Ohta et al., 2009; Wikoff et al., 2009), our HRM method is more robust. Overall, these data indicate the utility of applying this approach for bovine plasma and urine metabolomics.

Plant ergot alkaloid levels are influenced by numerous abiotic factors (Guerre, 2015), and E+ EA levels here were slightly lower than previous summer studies (Hill et al., 2000). This is expected, however, as warming increases E+ alkaloid content (McCulley et al., 2014). Total urinary EA concentration increased after 1 day of grazing, peaked on day 14, and remained elevated. The trend in urinary alkaloid excretion was consistent with previous studies (Ayers et al., 2009; Hill et al., 2000; Stuedemann et al., 1998), indicating animal's response to E+ grazing from this experiment is typical. Based on the grass and urinary EA levels and the reported relationship between EA excretion and weight gains (Hill et al., 2000), the effect of E+ grazing in the current study on average daily gain would have been modest.

HRM can provide metabolite-specific information of the EA and similar/related compounds in the plasma and urine. The largest number of EA identified in the urine were lysergic acid amides and ergoline derivatives, peaking after 2 days of grazing and, consistent with previous findings that ergot alkaloids less than 450 Da are excreted in the urine (Stuedemann et al., 1998); only after 14 days were urinary ergopeptides detected. In the plasma, both lysergic acid amides/ergoline derivatives and ergopeptides were identified throughout the grazing period; slightly greater numbers were detected after

14 and 28 days of grazing, potentially attributed to plasma and/or tissue accumulation of EA resulting from continuous exposure (Klotz, 2015).

Our metabolomics approach was optimized for extraction of the entire metabolome, not specifically EA, and this is one potential reason for the lack of plasma ergovaline (Klotz and Nicol, 2016; Schultz et al., 2006) or urine lysergic acid (Lodge-Ivey et al., 2006; Schultz et al., 2006) identification. Rapid ergovaline metabolism and distribution to liver and kidney tissues also impedes its identification in the plasma (Ayers et al., 2009; De Lorme et al., 2007; Zbib et al., 2014). Besides using a broad, non-ergot alkaloid focused extraction method, some EA metabolites might not have been identified because of the lack of matching MS/MS spectra in existing reference metabolomics databases. Nevertheless, we putatively identified previously unreported EA and EA metabolites in the plasma and urine of E+ steers. The detection of hydrogenated and hydroxylated EA, i.e., dihydroergocryptine, hydroxydihydroergotamine, is potentially indicative of detoxification attempts by E+ animals, as increased polarity and hydrophilicity facilitate EA excretion and, potentially, decrease toxicity (Mulac et al., 2011); increased plasma EA metabolite numbers after 14 and 28 days of grazing potentially indicate lowered rates of excretion (Berde and Stürmer, 1978). Interestingly, another unreported metabolite, pergolide sulfone, is a secondary metabolite of pergolide produced by microorganisms (Smith et al., 1983) and its increase in the urine may indicate increased rumen microbiome metabolic activity of E+ steers. Using HRM to identify novel EA, their structures and biotransformation, followed by verification with targeted approaches is fundamental to our greater understanding of EA in general and of FT.

No significant differences were seen in plasma catecholamine levels at any time point. However, the trend for increased plasma DA after 28 days on E+ is in line with previous findings in sheep (Henson et al., 1987). The urine catecholamines DA and E followed biphasic patterns. Of note, pergolide, a D-2 receptor agonist, has biphasic effects on sleep and wakefulness patterns (Monti et al., 1988), which are dysregulated in E+ animals (Perumbakkan et al., 2007). Here, the secondary metabolite of pergolide, pergolide sulfone, was detected in the urine of E+ steers. Interestingly, in mares fed E+ seed-based diets,

decreased plasma DOPAC levels were reported (Youngblood et al., 2004); here, DOPAC levels were not affected in the plasma, but were decreased in the urine in E+ steers, with more prominent effects occurring in the first two weeks of grazing.

Tryptophan metabolism was a major E+ target, with one portion of this pathway, tryptophan metabolism through serotonin, being affected in opposite directions in the plasma and urine. For example, while melatonin was not directly measured, metabolites derived from it were increased in the urine (i.e., AFMK) and decreased in the plasma. Decreased plasma melatonin in E+, which would be consistent with the findings here, has been reported (Porter et al., 1993). AFMK is the product of the reaction between melatonin and reactive oxygen species (ROS; de Almeida et al., 2003; Hardeland et al., 1995; Tan et al., 2000), and previous reports found widespread decreased antioxidant defense mechanisms due to E+ (Lakritz et al., 2002; Settivari et al., 2008; Zbib et al., 2014, 2015). Thus, increased AFMK may indicate early compensatory mechanism utilizing melatonin for the mitigation of E+-induced oxidative stress.

One putative metabolite that was impacted in E+ steers and is of potential importance to the pathophysiology of FT is AA-5-HT. AA-5-HT is a selective fatty acid amide hydrolase (FAAH) inhibitor that was consistently increased in the E+ plasma. AA-5-HT-induced FAAH inhibition increases four-fold when oxidized by CYP450's (Siller et al., 2014). AA-5-HT is present in the GI tract of multiple species (Verhoeckx et al., 2011) and it was recently detected in bovine and human brain and thymus tissues, indicating that it becomes systemic (Siller et al., 2014). AA-5-HT is sensitive to dietary fat (Verhoeckx et al., 2011) and decreased uptake of VFA (Dijkstra et al., 1993; Foote et al., 2013; Foote et al., 2014), along with significant increases in putative plasma glycerophospholipid metabolites seen herein, may play a role in decreased nutrient utilization, resulting in lowered weight gains which is a finding consistently reported in chronic E+ grazing (Oliver, 1997; Strickland et al., 2009). It is hypothesized that AA-5-HT increases in tissues with higher levels of serotonin (Verhoeckx et al., 2011);

increased serotonin in the central nervous system (Porter, 1995) and increased serum tryptophan levels (Oliver et al., 2000b) are all results of E+ grazing, which is consistent with the increases in AA-5-HT.

As stated, AA-5-HT is a selective FAAH inhibitor and FAAH predominantly cleaves one of the two major endocannabinoids, anandamide (Kathuria et al., 2003). AA-5-HT-induced increased levels of anandamide decrease gut motility (Bashashati et al., 2012; Capasso et al., 2005), consistent with gut hypomotility found in FAAH knockout mice (Cravatt et al., 2001). Of note, Egert et al. (2014b) recently showed that intravenous or intraruminal injections of an ergovaline and ergovalinine mixture did not reduce rumen motility, suggesting that either other EA affect gut motility or, alternatively, the hypothesized impact of AA-5-HT on food intake is independent of its effects on gut motility as plasma anandamide is also associated with better feed efficiency, at least in feedlot steers (Artegoitia et al., 2016). The role of AA-5-HT in FT warrants further investigation.

Multiple putative plasma glycerophospholipids were affected across all time points (more so later in the study), indicating significant effects of E+ on lipid metabolism. Accumulation of EA in adipose tissue (Realini et al., 2005) could potentially be responsible for the likely disruption of lipid metabolism due to E+ grazing, as it has been suggested recently (Klotz, 2015). Glycerol is an important energy source in ruminants and is a VFA precursor (Nafikov and Beitz, 2007); the increased levels of putative plasma glycerol metabolites (i.e., phosphatidylinositol and 3-beta-D-galactosyl-sn-glycerol) identified could indicate perturbed glycerol utilization. Further, acetate, a major acetyl-CoA source, and glucose are both main carbon sources for fatty acid synthesis in ruminants (Ballard and Hanson, 1967). Glucose is also the main substrate in intramuscular adipose tissue (Smith and Crouse, 1984), and increased plasma phospholipids are one potential utilization route of these important energy sources. Glycerophospholipids are also major structural lipids and are integral, alongside cholesterol, for membrane structures (Cooper, 2000). Lowered cholesterol has been reported in E+ grazing beef cattle (Oliver et al., 2000a) and this, coupled with an increase in plasma phospholipids and their precursors, could be an indication of decreased membrane integrity, including of muscle cells.

Interestingly, delayed (14 and 28 days) increase of E+ steers' plasma phosphocholine was observed. Phosphocholine is a precursor and a constituent of the platelet activating factor (PAF; McIntyre et al., 1999; Snyder, 1990). Also, phosphocholine, phosphorylcholine, and phosphatidylethanolamine, are intermediates in the synthesis of phosphatidylcholine (DeLong et al., 1999). This significant increase of phosphatidylcholine and PAF precursors involved in the most common PAF biosynthetic pathway could be partially responsible for/reflective of early FT signs, such as smooth muscle cell hyperplasia, endothelial cell damage (Strickland et al., 2009), and enhanced platelet aggregation (Oliver, 1997, 2005), when considered alongside elevated von Willebrand factor, angiotensin converting enzyme (Oliver, 1997), and endothelin (Strickland et al., 1994).

Other putative metabolites of interest affected by E+ grazing include cortolone-3-glucuronide, tetrahydroaldosterone-3-glucuronide, and 12-hydroxyeicosatetreanoic acid (12-HETE). Increased urinary tetrahydroaldosterone-3-glucuronide has been proposed as a predictive urinary biomarker for primary aldosteronism (Abdelhamid et al., 2003). In support, Piper (1988) reported increased basal levels of aldosterone in E+-exposed sheep. 12-HETE has been used as a urinary biomarker of hypertension and platelet activation (Sterz et al., 2012; Yeung and Holinstat, 2011) and has vasoconstrictive properties in the kidneys, where it increases in response to angiotensin II (Ma et al., 1991; Yiu et al., 2003), which is likely increased in FT (Oliver, 1997, 2005).

The plasma and urine networks indicate significant primary and secondary interactions between E+ grazing and target metabolites. Most overlapping features in the plasma networks were putatively identified as glycerophospholipids, tryptophan metabolites, and steroid hormone metabolites. Overlapping features in the urine networks contained tryptophan, steroids, and unidentified metabolites.

Overall, data from this study validate HRM as a novel, robust method for metabolic profiling of bovine plasma and urine, with the plasma metabolome being more appropriate for determining global effects of E+ grazing. The urine metabolome might be more useful for early detection of biomarker metabolites of exposure to and effects of ergot alkaloids that point to affected pathways and processes.

The implementation of HRM allows for sensitive detection of metabolic aberrations in cattle plasma and urine, which can point to molecular changes associated with pathologies specific to cattle and allow for targeted treatment strategies. Data presented here provide evidence that E+ grazing perturbs, among others, tryptophan, tyrosine, and glycerophospholipid metabolism. The data also provide putative metabolite-based explanation(s) for complications seen in FT. AA-5-HT in the plasma and tetrahydroaldosterone-3-glucuronide and 12-HETE in the urine are metabolites of possible use as biomarkers, but require further investigation. Studies of the nature of the one described here are imperative for potential exploration of new treatment and diagnostic strategies for FT.

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Tables

Table 1. Total number of unique m/z features detected in bovine plasma and urine of Angus steers grazing non-toxic (Max-Q) or toxic (E+) tall fescue for up to 28 days when analyzed using anion exchange (AE) or reverse phase (C18) LC coupled with apLCMS are presented.

Unique features detected				
Biological matrix	Plasma		Urine	
LC column	AE	C18	AE	C18
Unique features	9,403	11,505	6,478	6,612

Table 2. Number of significantly different ($P < 0.05$) m/z features between the Max-Q and E+ groups in bovine plasma and urine of Angus steers grazing non-toxic (Max-Q) or wild-type toxic tall fescue (E+) when analyzed using AE or C18 LC coupled with apLCMS after 1, 2, 14, and 28 days of grazing.

Matrix	Plasma								Urine							
LC column	AE				C18				AE				C18			
Days on pasture	1	2	14	28	1	2	14	28	1	2	14	28	1	2	14	28
Max-Q vs E+ ($P < 0.05$)	267	192	400	320	315	189	388	523	73	234	91	59	138	264	166	66

Table 3. Number of putative ergot alkaloid metabolites detected using metabolomics (AE and C18 columns combined) in the (A) plasma and (B) urine of Angus steers grazing toxic tall fescue (E+) for 1, 2, 14, or 28 days and the total number of ergot alkaloids identified from across all time points.

3A Plasma					
Days on pasture	1	2	14	28	Total
Ergoline derivatives and lysergic acid amides	4	0	3	3	10
Ergopeptides	2	1	5	5	13

3B Urine					
Days on pasture	1	2	14	28	Total
Ergoline derivatives and lysergic acid amides	1	5	2	1	9
Ergopeptides	0	0	2	0	2

Table 4. Names of ergot alkaloid metabolites identified after Metlin querying, separated by biological matrix and detection column (anion exchange [AE], or reverse phase [C18]), and grouped by their basic ring structure.

Putative ergot alkaloids identified via Metlin				
Biological matrix	Plasma		Urine	
LC column	AE	C18	AE	C18
Ergoline derivatives/ lysergic acid amides	Ergine	Ergoline-1-carboxaldehyde, 8-(hydroxymethyl)-10-methoxy-6-methyl-, (8b)-	Nicergoline	Pergolide sulfone
	Dihydroisolysergic acid II	Ergonovine	Ergoline-8-methanol, 10-methoxy-1,6-dimethyl	
	N-Despropylpergolide	Methylergonovine	Ergonovine	
	Ergonovine	Pergolide sulfone	Methysergide__	
Ergopeptides_ —	Dihydroergocornine__	Dihydro- α -ergocryptine		Ergosine
	α -Ergocryptine	Dihydro- β -ergocryptine		
	Ergotamine	α -Ergocryptine		
		Dihydroergocornine		
		Dihydroergotamine		8'-Hydroxy-dihydroergotamine
		Ergocornine		
		8',10'-Dihydroxy-dihydroergotamine		
		8'-Hydroxy-dihydroergotamine		

Table 5. Selected putatively identified metabolites, detected using HRM (AE and C18 columns combined), in the plasma and/or urine of Angus steers that were significantly impacted during the 28-day toxic (E+) fescue grazing (all sample dates [1, 2, 14, and 28 days] combined). The group or pathway that the metabolites belong to and their respective KEGG or Metlin IDs, as well as the direction of the change, are included.

Group/Pathway	Metabolite name	KEGG ID (or Metlin)	Biological Matrix	Direction of Change
Glycerophospholipid	Phosphocholine (Phosphorylcholine)	C00588	Plasma	Increased
	Phosphatidylinositol	C00626	Plasma	Increased
	Phosphatidylethanolamine		Plasma	Increased
Glycerophospholipid & galactose metabolism	3-beta-D-Galactosyl-sn-glycerol	C03692	Plasma	Increased
Trace amines	Phenylethanolamine	59 (Metlin)	Urine	Increased
	Octopamine	C04427	Urine	Increased
Tryptophan metabolism	5-methoxytryptamine	C05659	Plasma	Increased
	5-hydroxyindolacetyl glycine	C05832	Plasma	Decreased
	Tryptamine	C00398	Plasma	Increased
	Indole-3-thiohydroximate	C16516	Plasma	Increased
	3-dehydroshikimate	C02637	Plasma	Increased
	AFMK	C05642	Urine	Increased
	5-methoxyindoleacetate	C05660	Urine	Decreased
	5-hydroxyindolacetaldehyde	C05634	Urine	Decreased
Tyrosine metabolism	Tyramine	C00483	Plasma, Urine	Both
Fatty acyls	Arachidonyl serotonin (AA-5-HT)	63067 (Metlin)	Plasma	Increased
Sterol lipids	Cortolone-3-glucuronide	61643 (Metlin)	Urine	Increased
	Tetrahydroaldosterone-3-glucuronide	61672 (Metlin)	Urine	Increased
Eicosanoids	12-hydroxyeicosatetreanoic acid (12-HETE)	75018 (Metlin)	Urine	Increased
Steroid hormone	Chlormadinone acetate	C12729	Urine	Increased

Figure Legends

Figure 1. Urinary ergot alkaloid concentration detected via ELISA after 1, 2, 14, and 28 days on pasture for steers grazing non-toxic endophyte-infected tall fescue (Max-Q; n=4) versus steers grazing toxic wild-type endophyte-infected tall fescue (E+; n=4). * indicates significant differences between treatment groups within a time point, ^{a,b} indicate differences within fescue treatment due to duration of grazing ($P < 0.05$). Data are presented as $\bar{x} \pm \text{SEM}$.

Figure 2. Internal standard- (epinine) and creatinine-normalized urinary (A) dopamine (DA), (B) epinephrine (E), (C) norepinephrine (NE), and (D) 3,4-dihydroxyphenylacetic acid (DOPAC) levels (ng/mg creatine) after 1, 2, 14, and 28 days of steers grazing non-toxic endophyte-infected tall fescue (Max-Q; n=4) versus steers grazing wild-type toxic endophyte-infected tall fescue (E+; n=4). Data are presented as $\bar{x} \pm \text{SEM}$. * Indicates significant differences ($P < 0.05$).

Figure 3. Venn Diagrams presenting the significantly different ($P < 0.05$) m/z features due to toxic (E+) fescue grazing for all sample time points (1, 2, 14, and 28 days) for bovine plasma analyzed with (A) AE and (C) C18 LC and bovine urine analyzed with (B) AE and (D) C18 LC.

Figure 4. Volcano plots representing HRM-derived unique features due to toxic (E+) fescue grazing after 28 days of grazing in the plasma analyzed with (A) AE and (B) C18 LC and the urine after 2 days of grazing analyzed with (C) AE and (D) C18 LC.

Figure 5. PLS-DA plots (C18 column) for bovine plasma (A) containing all time points and (B) after 28 days of grazing; PLS-DA plots (C18) for bovine urine (C) containing all time points and (D) after 2 days of grazing non-toxic endophyte-infected tall fescue (Max-Q; n=4) or wild-type toxic endophyte-infected tall fescue (E+; n=4).

Figure 6. Heat maps representing apLCMS unique features (AE column) that were significantly different ($P < 0.05$) between the Max-Q and E+ groups for (A) plasma after 28 days of grazing and (D) urine after 2 days of grazing. Actual steer number and treatment (Max-Q vs E+) is indicated below the map. Neighboring animals ($n=2$) within the heat maps are from the same paddock (2 per paddock; indicated by black and red underlines for the Max-Q and E+ treatment groups, respectively).

Figure 7. Targeted complete Pearson correlation-based network ($|r| > 0.5$; FDR = 0.05) for bovine plasma metabolome of Angus steers grazing wild-type toxic (E+) fescue for 28 days (C18 column data). Numbers indicate the m/z and retention times of putative metabolites. Key anchor metabolite names are included. Red = serotonin metabolite, purple = indole metabolite, green = tyrosine metabolite, orange = ergot alkaloid metabolite.

Figure 8. Putative significantly different tryptophan pathway metabolites in the (A) plasma and (B) urine metabolomes of Angus steers grazing non-toxic (Max-Q) and wild-type toxic (E+) fescue. Red boxes = significant decreases, green boxes = significant increases, and blue boxes = significant increases or decreases that are time-dependent caused by E+ grazing. Number of arrows indicate individual metabolic steps. ‘?’ indicates potential source of tryptophan.

Total urinary ergot alkaloids

A**DA** **B****E** **C****NE** **D****DOPAC**

Plasma

A

AE

Urine

B

AE

C

C18

D

C18

A Plasma C18 All Time Points PLS-DA Plot

B Plasma C18 28 Day PLS-DA Plot

C Urine C18 All Time Points PLS-DA Plot

D Urine C18 2 Day PLS-DA Plot

Plasma AE 28 Days

Urine AE 2 Days

A

B