


# Serum metabolites associated with feed efficiency in black angus steers

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Received: 29 June 2017 / Accepted: 4 October 2017 / Published online: 27 October 2017  
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## Abstract

**Introduction** Improving feed utilization in cattle is required to reduce input costs, increase production, and ultimately improve sustainability of the beef cattle industry. Characterizing metabolic differences between efficient and non-efficient animals will allow stakeholders to identify more efficient cattle during backgrounding.

**Objectives** This study used an untargeted metabolomics approach to determine differences in serum metabolites between animals of low and high residual feed intake.

**Methods** Residual feed intake was determined for 50 purebred Angus steers and 29 steers were selected for the study steers based on low versus high feed efficiency. Blood samples were collected from steers and analyzed using untargeted metabolomics via mass spectrometry. Metabolite data was analyzed using Metaboanalyst, visualized using orthogonal partial least squares discriminant analysis, and p-values derived from permutation testing. Non-esterified fatty acids, urea nitrogen, and glucose were measured using commercially available calorimetric assay kits. Differences in metabolites measured were grouped by residual feed

intake was measured using one-way analysis of variance in SAS 9.4.

**Results** Four metabolites were found to be associated with differences in feed efficiency. No differences were found in other serum metabolites, including serum urea nitrogen, non-esterified fatty acids, and glucose.

**Conclusions** Four metabolites that differed between low and high residual feed intake have important functions related to nutrient utilization, among other functions, in cattle. This information will allow identification of more efficient steers during backgrounding.

**Keywords** Cattle · Metabolomics · Feed efficiency · Serum analysis

## 1 Introduction

Improving the feed efficiency of cattle is vital to increase the amount of beef produced per unit of feed, thus increasing production and decreasing input costs for producers in the United States. Feed is the largest cost to producers in the beef industry, representing approximately 60% of overall cost of production (Holmgren and Feuz 2015; Norton 2005). As resources become more limited and feed costs increase, the overall cost of production is likely to grow in subsequent decades (Lawrence et al. 2008). Therefore, the selection and optimization of economically important phenotypes, such as feed efficiency, must be evaluated.

Feed efficiency is moderately heritable (Berry and Crowley 2013), and genetic selection has been used to improve the efficiency of beef herds (Arthur et al. 2001). However, non-genetic factors such as the gut microbiome (Myer et al. 2017) and the environment (Mader and Davis 2004) also contribute to efficiency. Additionally, feed intake and body

**Electronic supplementary material** The online version of this article (doi:10.1007/s11306-017-1282-z) contains supplementary material, which is available to authorized users.

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weight gain, key components of efficiency, are themselves polygenic traits with complex patterns of inheritance (Elzo et al. 2012). Methods beyond genetic selection are therefore needed to optimize efficiency in beef cattle. Biochemically, efficiency results from composite changes in metabolism of macronutrients that increase the yield of energy from feed. Markers of this variation in metabolism that results in difference in efficiency could therefore be used as phenotypic traits for selection. However, relatively little is known at the molecular level about the basis for efficiency.

Metabolomics is a tool that produces a snapshot of cellular metabolism by comprehensively profiling metabolite abundance in biological samples. Accordingly, metabolomic profiling provides insight into nutrient utilization in humans and animals. It provides finer resolution of factors affecting phenotypic variation in growth and physiological parameters (Fontanesi 2016). Because metabolites are the result of combined endogenous and exogenous production, metabolomic studies reveal information beyond that provided solely from genetics or genomics, revealing relationships between animal genetics and physiological phenotypes (Fontanesi 2016). With respect to beef cattle, metabolomics is a dual-purpose tool that can both increase the fundamental understanding of efficiency and identify metabolites that are potential biomarkers for selection.

While previous studies have focused on phenotypic or genetic selection for feed efficient animals, understanding molecular-level changes that distinguishes animals that utilize feed more efficiently could provide valuable insight for the selection of efficient animals. In this study, we hypothesized that steers that differ in feed efficiency would have different levels of metabolites associated with nutrient utilization. The objective was to determine serum metabolites that differed between low and high feed efficient animals, utilizing untargeted metabolomics.

## 2 Materials and methods

This study was approved and carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville.

Fifty purebred Angus steers were used in this study. Steers from the University of Tennessee Institute of Agriculture Plateau Research and Education Center (precht and Williams 2012) in Crossville, TN were 7 months of age and weighed  $264 \pm 2.7$  kg at the start of the trial. Two weeks after weaning, steers were acclimated in the GrowSafe feeding system (GrowSafe Systems Ltd., Airdrie, Canada) for 10 days prior to measuring feed intake. Steers were fed a step up receiving diet for approximately 14 days before receiving a growing ration (11.57% crude protein and 76.93% total digestible nutrients on a dry matter basis) with 28 mg monensin/kg

DM. After the acclimation period, body weight (Krysl and Hess 1993) was measured at 7 day intervals and feed intake was continuously measured using the GrowSafe© system for a 60 day feed efficiency trial. As a measure of feed efficiency, at the end of the feeding period, steers were ranked by residual feed intake (RFI) based on performance and feed intake measured from day 0 to day 60 (Koch et al. 1963). The average value of RFI (actual dry matter intake (DMI) vs. expected DMI) and standard deviation (Elzo et al. 2012) were calculated for individual animals and were divided into two groups of low ( $n = 14$ ), and high ( $n = 15$ ) RFI. High RFI was defined as  $\text{RFI} \geq 0.5$  SD above the mean; and Low RFI was  $\text{RFI} \leq -0.5$  SD below the mean.

Weekly, approximately 9 mL of blood was collected from the coccygeal vein into serum separator tubes (Corvac, Kendall Health Care, St. Louis, MO) and centrifuged at 4 °C for 20 min at 2000xg. Serum was transferred via aspiration to plastic tubes and frozen at -80 °C for further analysis.

### 2.1 LC-MS analysis

Serum samples (50  $\mu\text{L}$ ) from each steer were extracted for metabolomic analysis using 0.1% formic acid in acetonitrile:water:methanol (2:2:1), as described previously (Kamphorst et al. 2011). Metabolites were separated using a Synergy Hydro-RP column (100  $\times$  2 mm, 2.5  $\mu\text{m}$  particle size). Mobile phases consisted of A: 97:3 H<sub>2</sub>O:MeOH with 11 mM tributylamine and 15 mM acetic acid and B: MeOH. The gradient consisted of the following: 0.0 min, 0% B; 2.5 min 0% B; 5.0 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B, and 25 min, 0% B. Flow rate was set to a constant 0.200 mL/min and the column temperature was kept at 25 °C. The autosampler tray was kept at 4 °C and 10  $\mu\text{L}$  of sample was injected into the Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA). Electrospray ionization was used to introduce the samples into an Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Waltham, MA), using an established method (Kamphorst et al. 2011; Lu et al. 2010). Representative extracted ion chromatograms (EIC) and compound list with mass accuracies and retention times are available in Supplementary Information (Supplementary 1; Supplementary 2).

### 2.2 Data analysis

Raw files obtained from Xcalibur MS software (Thermo Electron Corp., Waltham, MA) were converted into the mzML format using ProteoWizard (Chambers et al. 2012). The converted files were imported into MAVEN (Metabolomic Analysis and Visualization Engine for LC-MS Data), a software package (Clasquin et al. 2012). Peaks for the known metabolites were picked in MAVEN, which

automatically performs non-linear retention time correction and calculates peak areas across samples, using a preliminary mass error of  $\pm 20$  ppm and retention time window of 5 min. The UTK Biological and Small Molecule Mass Spectrometry Core (BSMMS) has replicated and expanded the method of Rabinowitz and coworkers (Lu et al. 2010) and final metabolite annotations were made using a library of 263 retention time-accurate  $m/z$  pairs taken from MS1 spectra. The annotation parameters have been verified previously with pure standards as part of establishing the method. For a metabolite to be annotated as a known compound, the eluted peak had to be found within 2 min of the expected retention time, and the metabolite mass had to be within  $\pm 5$  ppm of the expected value. Metabolite identities were confirmed using the MAVEN software package (Clasquin et al. 2012), and peak areas for each compound were integrated using the Quan Browser function of the Xcalibur MS Software (Thermo Electron Corp., Waltham, MA). Metabolomics data were pre-processed and analyzed using Metaboanalyst (Xia et al. 2015). Variables with missing values for  $> 20\%$  of samples were removed from the dataset prior to statistical analyses. Missing data values were imputed using K-Nearest Neighbors (Stacklies et al. 2007). Peak areas were normalized by median values, transformed logarithmically, and scaled using Pareto scaling prior to statistical testing. Metabolites differing significantly ( $P < 0.05$ ) between low- and high-RFI steers were identified using  $t$ -test. Multiple testing was addressed by setting a false discovery rate of 5%, using the method of Benjamini-Hochberg (Benjamini and Hochberg 1995). Orthogonal partial least squares discriminant analysis (O-PLS-DA) was used to visualize separation between the two groups of steers based on serum metabolite profiles. The metrics  $R^2Y$  and  $Q^2$  were used to evaluate the fit and prediction power, respectively, of the O-PLS-DA model. Validity of model estimates was evaluated using permutation testing (van Velzen et al. 2008). Sample labels were randomly assigned 1000 times, and new models were fitted and estimates of  $R^2Y$  and  $Q^2$  were calculated for each random permutation. Values of  $R^2Y$  and  $Q^2$  from the original model were compared to the distribution of values from the permutations to calculate empirical  $p$ -values for  $R^2Y$  and  $Q^2$ .

Serum samples were analyzed using a 96-well EPOCH 2 microplate reader (BioTek Instruments, Winooski, VT) with commercial kits for non-esterified fatty acids (NEFA; Wako Chemicals, Richmond, VA; sensitivity of 0.01 mmol/L), glucose (Thermo Electron Corp., Waltham, MA; sensitivity of 0.3 mg/dL), and serum urea nitrogen (SUN; Thermo Electron Corp., Waltham, MA; sensitivity of 2.0 mg/dL). The intra- and interassay CV were, respectively, 4.08 and 3.73% for serum NEFA, 0.53 and 4.31% for serum glucose, 0.30 and 0.35% for SUN. Measurements were tested for normality using the PROC UNIVARIATE command in SAS 9.4 (SAS

Institute, Cary, NC) and did not follow normal distribution. Non-parametric one way ANOVA was calculated using Wilcoxon rank-sum test in SAS 9.4. Statistical significance was set at  $P \leq 0.05$ .

### 3 Results

A total of 109 known metabolites were identified in serum samples from low- and high-RFI steers. Multivariate analysis (O-PLS-DA) was used to visualize the extent to which serum metabolomes discriminated steers with low versus high RFI. As shown in Fig. 1, steers tended to separate according to RFI, based on lack of overlap of Hotelling's 95% confidence intervals. Statistically significant differences in metabolite abundance between the two groups of animals were identified by  $t$ -test. After controlling for multiple testing, four metabolites (homocysteine, pantothenate, carnitine and glutamate) differed significantly between low- and high-RFI steers ( $FDR < 0.05$ ; Fig. 2). Each of the four metabolites was present at lower levels in serum of high- versus low-RFI animals (Table 1).

Circulating serum glucose, NEFA, and SUN concentrations did not differ significantly between low- and high-RFI steers ( $P \geq 0.05$ ; Table 2).

### 4 Discussion

Understanding the mechanisms driving the variation in feed efficiency in animals of similar genetic backgrounds could lead to substantial innovations in agriculture. Complex phenotypes such as feed efficiency are attributed to many confounding factors that are not completely understood. We hypothesized that steers differing in feed efficiency during a backgrounding phase would produce different levels of metabolites associated with nutrient utilization. Using LC-MS to perform untargeted metabolomics, it was found that metabolites differed between low- and high-RFI steers were pantothenate, carnitine, homocysteine, glutamine

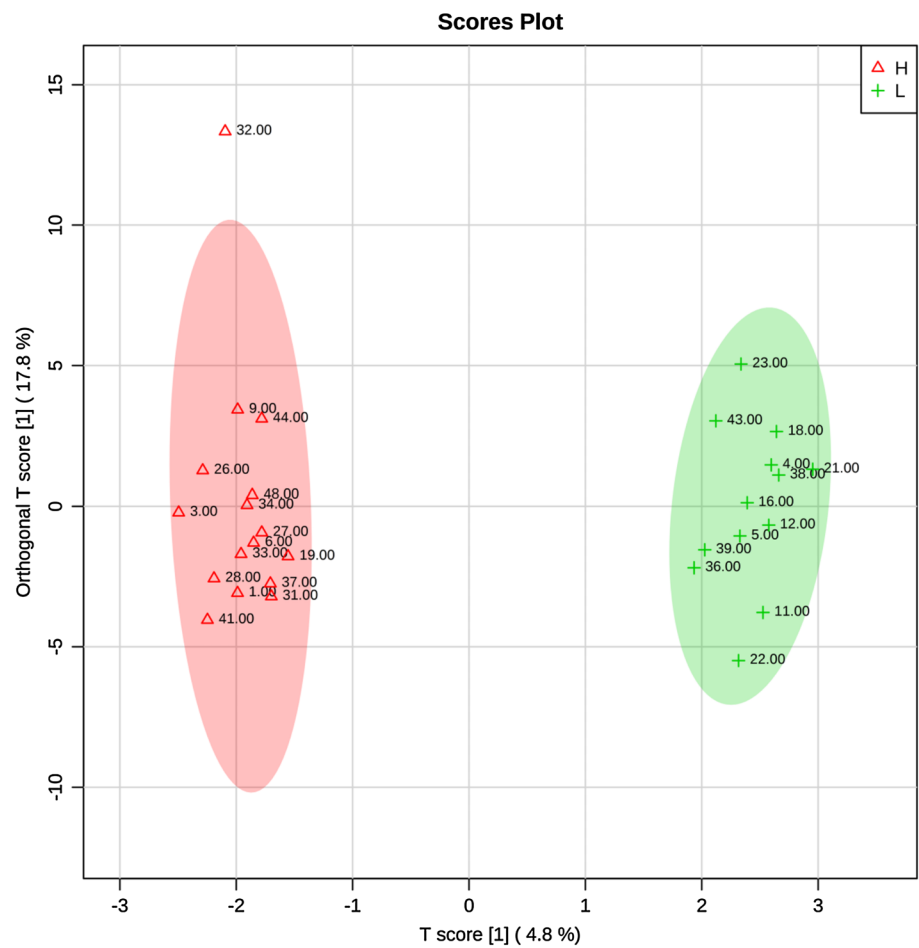
**Table 1** Blood serum metabolites associated with high- and low-RFI steers at week 10

Metabolite	FDR P value <sup>a</sup>	Fold change (high/low RFI) <sup>b</sup>
Pantothenate	0.009586	0.7635
Homocysteine	0.01677	0.7610
Glutamine	0.01707	0.7496
Carnitine	0.08253	0.7220

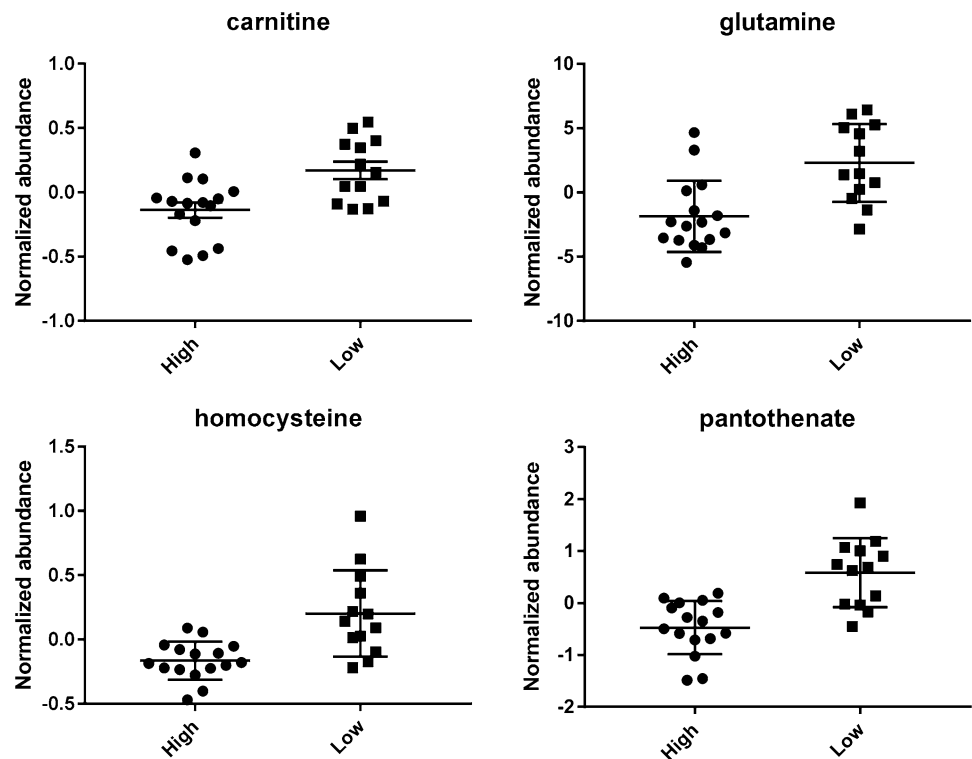
<sup>a</sup>FDR controlled by the method of Benjamini-Hochberg

<sup>b</sup>Based on normalized data

**Fig. 1** 2D score plot from orthogonal PLS-DA of serum metabolomes from low (green plus symbol) and high (red triangle) RFI steers. Model fitting based on  $R^2Y$  (0.98) and  $Q^2$  (0.41) was validated using permutation testing ( $P=0.004$  and 0.058, respectively) using 1000 permutations. Ellipses represent 95% confidence intervals



**Fig. 2** Dot plot representation of carnitine, glutamine, homocysteine and pantothenate levels in low- and high-RFI steers. Data values shown were normalized using median normalization and pareto scaling. Horizontal lines represent mean  $\pm$  standard deviation of normalized data



**Table 2** Concentrations of serum NEFA, glucose, and SUN

Metabolite	Low-RFI concentrations	High-RFI concentrations	P value
NEFA <sup>a</sup>	262.6 ± 15.24	263.9 ± 9.28	0.1970
Glucose <sup>b</sup>	47.48 ± 7.47	39.43 ± 3.47	0.2845
SUN <sup>b</sup>	2.14 ± 0.19	2.15 ± 0.20	0.4614

<sup>a</sup>Measured as mmol/L<sup>b</sup>Measured as mg/dL

(Table 1), while no differences were observed in circulating serum NEFA, SUN, or glucose concentrations between the two groups ( $P > 0.05$ ). Delineating discrepancies in metabolite production in steers of dissimilar feed efficiencies may provide critical information critical for understanding factors associated with diverse feed efficiency phenotypes.

Pantothenate, or vitamin B<sub>5</sub>, was greater in low-RFI steers. Ruminants get PA in several ways; via absorption directly from feed, endogenous synthesis, or microbial production (Wegner et al. 1940). PA is required by all living organisms as it is necessary for the production of coenzyme A (Ball 2006; Begley et al. 2001; Bender 2003; McGinn et al. 2004; Pietrzik et al. 2008). Coenzyme A is key in intermediary metabolism, necessary for many amino acid, carbohydrate, and fat metabolism reactions (Leonardi et al. 2005). This coenzyme typically acts as a carrier protein, binding to acetyl and acyl groups for carbohydrate and fat metabolism, respectively (Leonardi et al. 2005). Acetyl-CoA introduces an acetate group into the tricarboxylic acid (TCA) cycle, while acyl-CoA assists with transport of fatty acids into and out of mitochondria (Begley et al. 2001; Leonardi et al. 2005). Coenzyme A can also be esterified to propionate, the most common glucogenic precursor in healthy and normally functioning cattle (Yost et al. 1977). Propionyl-CoA is converted to succinyl-CoA through a series of enzymatic reactions, which can then enter the TCA cycle (Young 1977). Increased coenzyme A acts as an inhibitory agent in the formation of coenzyme A from PA, but carnitine can reverse this inhibition (Miller et al. 2001). Because carnitine was also greater in low-RFI steers, it may help to counteract the inhibitory processes of the increased concentrations of PA or coenzyme A (Miller et al. 2001). Ruminants are unique in that gluconeogenesis is the primary source of glucose in the ruminant (Freetly and Klindt 1996; Bergman et al. 1974; Young 1977), compared to the non-ruminant, which relies on direct glucose absorption from the diet (Nafikov and Beitz 2007; Young 1977). In amphibians and monogastric species, supplementation of PA results in increased muscle performance, though results are inconsistent (Litoff et al. 1985; Miller et al. 2001; Nice et al. 1984; Shock and Sebrell 1944; Smith et al. 1987). Although physiology of monogastric

and ruminant species differ greatly, increased concentrations of PA may increase muscle performance and growth of animals (Karasov and Douglas 2013).

Carnitine concentrations were elevated in low-RFI steers. Carnitine provides several important functions related to metabolism in the body, including import of long chain fatty acids (LCFA) into the mitochondria for  $\beta$ -oxidation via interaction with the coenzyme A attached to the fatty acid (Bremer 1983). Carnitine is also involved in controlling the acetyl-CoA to coenzyme A ratio, transporting long and medium chain fatty acids into the mitochondria, and mediating energy-associated molecules (Arslan 2006). Carnitine is synthesized endogenously, but several precursors and cofactors are required, including lysine, methionine, vitamin C, niacin, vitamin B12, choline, and reduced iron (Bremer 1983). Increasing carnitine in dairy cow hepatic cells stimulated LCFA  $\beta$ -oxidation in vitro (Drackley et al. 1991), and this has been demonstrated in vivo in other species, including beef cattle (Cetin et al. 2003; Greenwood et al. 2010). Carnitine supplementation in vivo also increased plasma glucose concentrations (Cetin et al. 2003; Greenwood et al. 2001). Increased carnitine and subsequent metabolism may result in more efficient nutrient utilization (Karisa et al. 2014). In our study, though no significant differences were reported between low- and high-RFI steers in serum glucose concentrations, low-RFI steers had numerically greater glucose concentrations than high-RFI steers. Greater carnitine levels may have led to increased concentration of glucose in the steers in this study, though additional studies should be conducted to confirm.

Low-RFI steers also had greater concentrations of homocysteine than high-RFI steers. Homocysteine is an intermediate metabolite in the interconversion pathway of methionine and cysteine and concentrations differ based on physiological need (Lehninger 1977). Homocysteine supplementation increases methionine synthase, the enzyme responsible for conversion of homocysteine to methionine, in vitro (Ortiou et al. 2004). Abomasal infusion of homocysteine increased plasma concentrations of methionine in wethers (Amos et al. 1974). Methionine is required for protein synthesis and other metabolic processes in ruminants (Lehninger 1977; Seymour 2016) and can be an indicator of energy balance in cattle (Pedernera et al. 2010). Methionine is usually the first limiting amino acid, particularly in growing steers (Titgemeyer and Merchen 1990; Richardson and Hatfield 1978). Though no differences were observed between low- and high-RFI steers in methionine or cysteine using the methods described in this study, greater concentrations of homocysteine may indicate increased metabolic reactions involving methionine as a methyl donor (Ditscheid et al. 2005) or cysteine. It could also indicate more substrate availability for methionine-dependent reactions (Amos et al. 1974; Ortiou et al. 2004).

Greater concentrations of glutamine were measured in low-RFI steers. In livestock species, the goal of production is to promote muscle synthesis to increase available animal protein for consumption. In growing ruminants, glutamine may act as an anabolic mediator, increasing muscle growth (Lobley et al. 2001); at the very least, it has been linked to decreased catabolism of muscle (Reecy et al. 1996). Glutamine synthetase, the enzyme responsible for synthesizing glutamine from glutamate and ammonia, increases during time of backgrounding and weaning in steers, but decreases during the finishing stage (Matthews et al. 2016). This could be indicative of decreased need for glutamate for muscle growth, which is responsible for approximately half of circulating glutamine (Matthews et al. 2016). Glutamine is a very potent cause of protein swelling, which leads to protein synthesis (Bequette 2003). Glutamine is also a gluconeogenic amino acid, and it has been hypothesized that greater circulating levels of some gluconeogenic amino acids may moderate apparent energy status of beef cattle, and affect feed intake (Karisa et al. 2014).

A lack of differences in glucose, NEFA, SUN was unexpected given their use as markers of health and nutrient status in cattle (Adewuyi et al. 2005; Gleghorn et al. 2004; Ørskov et al. 1999). Attempts to correlate NEFA, SUN, and glucose with other metabolites, such as carnitine, have yielded inconsistent results (Adewuyi et al. 2005; Greenwood et al. 2001; Kelly et al. 2010). Glucose is highly regulated in ruminants and is constantly being produced via gluconeogenesis (Young 1977). Glucose concentrations can remain the same in growing steers regardless of diet (Seal et al. 2007). The tight regulation of glucose in ruminants may explain the lack of differences found between low- and high-RFI steers in this study. NEFA concentrations are used to infer information about the nutrient status of cattle (Bowden 1971). NEFA in blood is a result of mobilization of adipose tissue, particularly during times of fasting or high energy requirements, such as lactation (Bowden 1971; Reid and Hinks 1962). However, because the steers used in this study were growing steers, they would have little need to mobilize adipose tissue and would have lower concentrations of NEFA in serum. SUN is used in ruminants and other production species as an indicator of protein intake (Preston et al. 1965), nitrogen utilization (Egan and Kellaway 1971; Kohn et al. 2005), and nitrogen intake (Nolan et al. 1970). It is currently regarded as one of the most effective measurements of protein status in ruminants (Herd 2000). In dairy cows, SUN concentrations increase after feeding (Miettinen and Juvonen 1990). Blood sample collections occurred prior to morning feeding in this study, which may partially account for the decreased concentrations in SUN.

Based on the findings of this study, Black Angus beef steers differing in feed efficiency exhibit varying levels of serum metabolites associated with nutrient utilization and

energy status. The majority of the metabolites that were found to be in greater blood circulation in low-RFI steers are associated with energy usage, including pantothenate, glutamine, carnitine, and homocysteine. In addition, these four metabolites are intimately related, as they either inhibit or facilitate nutrient metabolism reactions. Determining causes of divergence in the production of these metabolites may, in part, account for some of the variation in feed efficiency of growing Black Angus beef steers. Subsequent analyses utilizing different techniques, such as transcriptomics, could bridge the gap between correlation and causation. Additional analyses should also be conducted across breeds and species to determine metabolite biomarkers that may be unique to those animals. Further understanding of the mechanisms driving these trends will result in improved nutrient utilization, increased feed efficiency, and reduction of production costs.

**Acknowledgements** This study was supported by Ascus Biosciences, Inc. (Grant No. A17-0146-003) and USDA-NIFA Hatch/Multistate Project W2010- TEN00493—Integrated Approach to Enhance Efficiency of Feed Utilization in Beef Production Systems. The authors thank Ashley Honey and the staff at the Plateau Research and Education Center in Crossville, TN for their technical assistance.

#### Compliance with Ethical Standards

**Ethical approval** All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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