

The Equine Extension Expanse: Reaching the next generation of industry professionals by integrating applied metabolomics and a train-the-trainer approach

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ABSTRACT

The University of Tennessee Extension System and others land grant institutions strive to translate basic science into usable information for various outlets and use applied research to answer practical problems. Currently, the large population of emaciated horses continues to challenge the equine industry; however, little is known regarding the equine metabolome during emaciation and the changes that occur throughout nutritional rehabilitation. Ten emaciated horses underwent a refeeding process, during which blood samples were collected for a blood chemistry panel and metabolomics analysis. Statistical analysis was conducted using SAS v9.4 (Cary, NC). Significant differences in blood chemistry analytes and metabolite abundance were seen during the refeeding process. Potentially toxic compounds, analytes related to liver, kidney, and muscle function, as well as energy-related metabolites were also altered during the refeeding process. Alantoin ($p \leq 0.01$), phenylalanine ($p \leq 0.1$), and methionine ($p \leq 0.05$) were found to be significantly decreased in horses with higher BCS scores. Land-Grant Institutions and Cooperative Extension Services seek to disseminate information such as this to the general public. However, Extension agents differ in areas of expertise, leaving some counties with minimal ability to provide 4-H horse project members with sufficient content knowledge while agents in other counties are more well versed in equine-specific areas. The Tennessee regional and Eastern National 4-H Hippology contests were used to determine areas of knowledge deficiency. Nutrition, tack, selection, health, and breeds were categories identified as areas in which 4th – 12th grade youth lacked adequate knowledge and a training program and new curriculum was developed and delivered to county extension agents. Statistical analysis was conducted using SAS v9.4 (Cary, NC). Nutrition questions most often missed by senior and junior high youth ($k=7.8$, 51.94%; $k=7.5$, 44.22%) whereas junior youth missed training questions most frequently ($k=4.9$, 54.14%). Of the 5 topic areas of deficiency, selection questions were the lowest percentage missed by senior and junior high youth ($k=5.9$, 36.81%; $k=7.1$, 39.53%) whereas junior youth missed health questions least frequently ($k=4$, 39.87%). It was found that training status had no significant on scores from year to year. However, significant effects were found when comparing across question category ($p<0.0001$), age group

($p < 0.0001$), and year ($p < 0.0001$). Despite the lack of training effect, these findings still prove valuable when assessing performance upholding the extension mission of delivering science-based information to the next generation of industry professionals.

TABLE OF CONTENTS

CHAPTER I LITERATURE REVIEW: METABOLOMIC PROFILES IN STARVED LIGHT BREED HORSES DURING THE REFEEDING PROCESS	1
Literature Review.....	2
Unwanted Horses	2
Malnutrition in Equids:	2
Intro to Metabolomics:.....	3
Previous Finding in the Equine Metabolome:	4
Previous Research in Malnutrition Metabolomics:.....	4
CHAPTER II METABOLOMIC PROFILES IN STARVED LIGHT BREED HORSES DURING THE REFEEDING PROCESS.....	6
Abstract	7
Introduction.....	7
Materials and Methods.....	9
Equine Enrollment	9
Study Timeline and Sample Collection	9
Diet Formulation	12
Blood Chemistry Analysis	12
Metabolomics Extractions	15
Ultrahigh Performance Liquid Chromatography Mass Spectrometry High Resolution Mass Spectrometry (UHPLC-HRMS).....	15
Data Analysis	16
Results.....	17
Equine Refeeding Outcomes.....	17
Analysis of Blood Chemistry Panels	17
Fold Change Analysis of Metabolites Detected in Equine Subjects during the CCP and RPs	20
Differences of the Metabolic Profiles of Equine Subjects in the CCP vs. the RP	20
Effects of Subject-Specific Attributes on the Metabolome	23
Effects of Subject-Specific Attributes on the Metabolome	23
Discussion	25
Animal Origin and Source	25
Blood Chemistry Analysis Suggests Improved Biological Function during the RP	26
Potentially Toxic Metabolites and Indicators of Oxidative Stress Were in Lower Abundance during the RP	28
Metabolites Regulated by Skeletal Muscle Were Altered between the CCP and RP31	
Significant Differences Were Observed in Metabolites Related to Liver and Kidney Function during the CCP and RPs	33
The Plasma Metabolome Illuminates Differences in Equine Subject-Specific Attributes	34
Conclusions.....	35
REFERENCES	37

CHAPTER III	43
EXTENSION EDUCATION: VARYING THE APPROACH TO EDUCATION, ASSESSMENT, AND TRAINING	43
Introduction/Extension Overview	44
Education Strategies.....	44
Online Learning	45
Face-to-Face Learning	46
Blended Learning Models.....	47
Extension/Agriculture Education.....	48
Importance/Overview	48
Extension Agent Background	48
Previous Research Efforts on Educating Extension Personnel	49
Measuring Performance	51
Train-the-Trainer Models.....	52
Implications and Future Directions.....	54
CHAPTER IV USING YOUTH HIPPOLOGY CONTEST RESULTS AS A NEEDS ASSESSMENT AS A FRAMEWORK FOR A TRAIN THE TRAINER PROGRAM ..	55
Abstract	56
Introduction.....	57
Materials and Methods.....	58
Regional Hippology Contests	58
National Contest.....	60
Training Content Selection and Format.....	61
Participant Selection and Determination of Training Status.....	62
Final Data Set Analysis.....	62
Results.....	62
Summary of Info/Introduction	62
Effects Age Division, Category, Year, and Training Status	63
Comparison of Tennessee Regional Scores and Eastern National Scores.....	68
Discussion	68
Study Origin.....	68
Differences Between Age Divisions	70
Differences Between Question Categories	71
Differences Between Year and Training Status.....	72
Difference in Senior Scores Between State and National.....	73
Limitations	73
Implications and Conclusions	75
REFERENCES	76
VITA	79

LIST OF TABLES

Table 1. Available metadata for equine subjects enrolled in the refeeding program.	10
Table 2. Nutrient analysis of forage of diet components.	13
Table 3. Measured parameters for blood plasma and serum.	14
Table 4. Outcomes for equine enrolled in the refeeding program.	18
Table 5. Biological significance of metabolites observed to have significantly increased or decreased between the RP and the CCP.	29
Table 6. Number of correct (1) and incorrect (0) responses across all years, categories, and age divisions	64
Table 7. Comparison across question categories of likelihood to have a correct answer.....	65
Table 8. Comparison across age division of likelihood to have a correct answer.....	66
Table 9. Comparison across year of likelihood to have a correct answer.....	67
Table 10. Comparison of percentage of correct responses from National and State contests across question categories.....	69

LIST OF FIGURES

- Figure 1. Box plots of blood plasma data conveying the differences between blood during the CCP ($n = 68$) and the RP ($n = 15$). Asterisks represent statistical significance (p value $\leq 0.1 = *$; p value $\leq 0.05 = **$). LDH: lactate dehydrogenase (p value 0.06), U/L; ALP: alkaline phosphatase, U/L; ALB: albumin (p value 0.04), g/dL; BUN: blood urea nitrogen, mg/dL; GGT: gamma-glutamyl transferase, U/L; mg/dL; TRIG: triglycerides, mg/dL; AST: aspartate amino transferase (p value 0.09), U/L; TP: total protein, g/dL; GLUC: glucose, mg/dL; Phos: phosphate, mg/dL; ALT: alanine transaminase, U/L; CREAT: creatinine (p value 0.04)..... 19
- Figure 2. Heatmap showing increased or decreased abundance of detected metabolites organized by compound class between the RP ($n = 20$) and the CCP ($n = 68$; fold change of RP/CCP). Eight compounds exhibited significantly lower abundance while one compound showed significantly higher abundance during the RP. 21
- Figure 3. PLS-DA containing known metabolites and unidentified features detected during the CCP ($n = 68$) and RP ($n = 20$). The resulting plot shows separation between the groupings, indicating differences in the equine metabolomic profile during the CCP and the RP. 22
- Figure 4. PLS-DAs (left) of known metabolites and unidentified features detected in the metabolomes of all horses that were enrolled in the refeeding program grouped by diet (C vs. S), sex (mare vs. gelding), and age group (below 20 and 20 and above). Sample sizes used for PLS-DAs were as follows C: $n = 52$; S: $n = 36$; mare: $n = 59$; gelding: $n = 29$; below 20: $n = 45$; 20 and above: $n = 43$. Venn Diagram (right) displaying similarities and differences between features in each grouping. 24

CHAPTER I
LITERATURE REVIEW: METABOLOMIC PROFILES IN STARVED
LIGHT BREED HORSES DURING THE REFEEDING PROCESS

Literature Review

Unwanted Horses

The equine industry within the United States is plagued with issue of a growing number or undesired or “unwanted” horses and other equids. (Schneider et al. 2021). Approximately 138,000 horses and other equids are deemed “unwanted” each year across the country (Lenz 2009). Often, these unwanted animals are geriatric, incurably lame, have behavioral issues, or are considered dangerous to their owners (Lenz 2009). Horses that are more difficult to market and sell, are physically unattractive, unathletic, or expensive to maintain often also become “unwanted” (Lenz 2009). Unfortunately, these undesirable animals are often neglected and become emaciated or starved due to lack of nutritional knowledge by the owner, lack of awareness of the animals’ maintenance cost, and in some cases, criminal neglect (Schneider et al. 2021). Furthermore, growing unwanted horse population coupled with the increased frequency of neglect cases and lack of owner knowledge results in a growing number of starved horses in the United States, thus taking a toll on the country’s equine industry.

Malnutrition in Equids:

Often, unwanted horses reach a state of emaciation or starvation as a result of owners lacking proper knowledge of equine nutrition and thus neglecting their animals (Schneider et al. 2021). Malnutrition is defined as a lack of nutrition due to inadequate or unbalanced intake of nutrients, or their impaired assimilation or utilization (Merriam-Webster). Severe malnutrition or starvation yields proteolytic and lipolytic responses that create harsh, negative physiological responses including refeeding syndrome, cachexia, serous atrophy, muscle contractions due to low calcium stores, and heart arrhythmia (Finn and Dice 2006). Additionally, malnutrition is a complex condition and there are a variety of factors that contribute to it. Thus, it can be difficult to truly label the severity of malnutrition of these animals. However, starvation in horses is typically classified using a low body condition score (BCS; < 3 on a 1 – 9 scale) (Henneke et al. 1983; Schneider et al. 2021). For this classification, the Henneke Body Condition Scoring system is

utilized to assess fat cover and overall nutritional status (Henneke et al. 1983). Often, this system is used to visually assess the nutritional status of equids.

As horses and other equids start to become emaciated or reach a poor nutritional status, their body condition score and overall body weight often begins to decrease (Kronfeld 1993). However, we cannot accurately determine the causes for these changes in body composition, metabolism, and overall health using the Henneke Body Condition Score system (Henneke et al. 1983). Poor nutritional status can result in an array of clinical health issues including dental deterioration, hoof issues, intestinal parasites, hyperlipemia, cancer, and other chronic diseases (Kronfeld 1993; Naylor et al., 1980). However, when severely emaciated animals are subjected to an abrupt refeeding process, they often die within the first few days due to the effects of refeeding syndrome (Kronfeld 1993). Therefore, careful attention must be paid in regard to quantity of feed given, nutritional value of feed, and diet composition when beginning the process of refeeding malnourished equids (Kronfeld 1993). Additionally, a large quantity of these horses are transported in and out of auction horses and sale barns across the country. Therefore, factors that influence nutritional status including genetics, background, predisposition to disease, and dietary history are often unknown within the unwanted and starved horse populations. Thus, a consistent evaluation method and knowledge of metabolic changes would prove to be crucial in contributing to the understanding of emaciated phenotypes and establishing a healthy body weight and condition to those animals.

Intro to Metabolomics:

Metabolomics provides a more concrete method to determining and evaluating biological systems within the body. Metabolomics is the global measurement of the combination and abundance of small molecule metabolites that makeup an organism's metabolome. The metabolome comprised of numerous small molecules that are created by or utilized within known existing metabolic pathways. Metabolomics provides a snapshot of an organism's physiological and provides a more quantitative, global measurement to supplement qualitative approaches like the Henneke Body Condition Scoring system (Henneke et al. 1983; Dettmer,

Aronov, and Hammock 2007). By analyzing the metabolome of emaciated horses, we have the potential to gather valuable information regarding which metabolic pathways are affected during a state of emaciation and how these pathways are altered as body condition scores change during the refeeding process. (Jiang et al. 2016).

Previous Finding in the Equine Metabolome:

The vast majority of studies investigating the equine metabolome are directed toward healthy thoroughbreds, pregnant mares, exercise physiology, and doping (Beachler et al. 2021; Escalona et al. 2015; Keen et al. 2022; Klein, Anthony, and McKeever 2021). One recently observed changes in the plasma metabolic composition in healthy pregnant mares when compared to mares induced with ascending placentitis (Beachler et al. 2021). It was found that there were 2 phases of significant changes in metabolites between inoculated and control mares (Beachler et al. 2021). A separate study sought to evaluate the metabolome of various body fluids in healthy thoroughbred racehorses to determine predominant metabolites to use as a baseline for future in regard to the equine metabolic profile (Escalona et al. 2015).

Previous Research in Malnutrition Metabolomics:

Other published articles have been geared toward identifying common practices in metabolomics and additional gaps withing the literature in regard to equine metabolomics in exercise and doping, indicating a significant lack of research on the equine metabolic profile and factors that affect metabolic pathways (Keen et al. 2022; Klein, Anthony, and McKeever 2021).

Research pertaining to the equine metabolome in regard to nutritional status, body condition score, and refeeding is virtually nonexistent. However, metabolomics research related to emaciation in other species is more common and can provide a foundation of knowledge when conducting future research on the equine metabolic profile. For example, it was observed that the metabolic profile of pigs undergoes significant changes during a status of malnutrition (Jiang et al. 2016). Additionally, it was found that evaluating the metabolome of emaciated children can aid in the prediction of mortality rates (Freemark 2015). It was also found that metabolomic

analysis can be used as an effective tool for identifying populations at risk for cancer cachexia (Yang et al. 2018). Despite the large variety of research and information within the field of metabolomics, none are centered around emaciated horses or the changes in their metabolic profile during the refeeding process. Research regarding the equine metabolome throughout stages of malnutrition and the refeeding process could provide insight into specific nutritional requirements and allow owners to make dietary adjustments accordingly.

CHAPTER II
METABOLOMIC PROFILES IN STARVED LIGHT BREED HORSES
DURING THE REFEEDING PROCESS

Abstract

The large population of emaciated horses continues to be an issue troubling the equine industry. However, little is known regarding the collection of equine metabolites (metabolome) during a malnourished state and the changes that occur throughout nutritional rehabilitation. In this study, ten emaciated horses underwent a refeeding process, during which blood samples were collected for a blood chemistry panel and metabolomics analysis via ultrahigh performance liquid chromatography–high resolution mass spectrometry (UHPLC-HRMS). Significant differences among blood chemistry analytes and metabolite abundance during the critical care period (CCP; Days 1–10 of rehabilitation) and the recovery period (RP; the remainder of the rehabilitation process) were observed. Potentially toxic compounds, analytes related to liver, kidney, and muscle function, as well as energy-related metabolites were altered during the refeeding process. The combination of blood chemistry and metabolomics analyses on starved equine during rehabilitation provide vital biological insight and evidence that the refeeding process has a significant impact on the equine metabolome.

Introduction

The equine industry continues to be plagued with a significant number of “un-wanted” horses and other equids (Schneider et al. 2021) with an estimated 138,000 equids deemed unwanted in the United States alone each year (Lenz 2009). For various reasons (including lack of nutritional knowledge by the owner, misalignment of expectation to actual cost of equine ownership, and in some cases, criminal neglect), many unwanted equids can become emaciated (Schneider et al. 2021). Despite the commonality of emaciated horses, there has been minimal research dedicated to the evaluation of their nutritional recovery and metabolic repair. The literature describes some studies on rehabilitating or refeeding the starved horse, yet these studies span only 10 to 15 days while the average refeeding period lasts for several months (Stull, Hullinger, and Rodiek 2003; Becvarova, Pleasant, and Thatcher 2009).

Malnutrition is diagnosed as a lack of nutrition due to inadequate or unbalanced intake of nutrients, or their impaired assimilation or utilization (Merriam-Webster). Severe malnutrition or

starvation yields proteolytic and lipolytic responses that can result in negative physiological issues including refeeding syndrome, cachexia, serous atrophy, muscle contractions due to low calcium stores, and heart arrhythmia (Finn and Dice 2006). The Henneke body condition scoring system is an important tool for rapid, qualitative assessment of nutritional status; however, this system cannot assess the underlying factors associated with respective changes in body composition, metabolism, and overall health (Henneke et al. 1983). Because malnutrition is a complex condition that is influenced by an array of factors, it is often difficult to determine and label the true state of malnutrition in equids, for which starvation is typically classified using a low body condition score (BCS; < 3 on a 1 – 9 scale) (Henneke et al. 1983; Schneider et al. 2021). Elements influencing nutritional status including genetics, background, predisposition to disease, and dietary history are often unidentified within the unwanted and starved horse populations. Thus, a consistent evaluation method coupled with an understanding of changes in metabolism is important to understanding emaciated phenotypes and, if possible, a recovery to a healthy body weight and condition.

A more quantitative approach to evaluating a biological system is that of metabolomics, the global measurement of the suite of small molecule metabolites that constitute an organism's metabolome. The metabolome is made up of numerous small molecules that are derived from or utilized by all known metabolic pathways. Metabolomics provides insight into an organism's physiological state (i.e., phenotype) and, thus, can serve as a quantitative, global measurement to supplement other qualitative approaches, such as the BCS system (Henneke et al. 1983; Dettmer, Aronov, and Hammock 2007). Investigating the metabolic profile of emaciated equids could provide information as to which metabolic pathways are affected during a state of malnutrition (Jiang et al. 2016).

At the present time, little research has been conducted in the field of equine metabolomics regarding nutritional rehabilitation. Current equine studies mainly focused on healthy thoroughbreds, pregnant mares, exercise, and doping (Beachler et al. 2021; Escalona et al. 2015; Keen et al. 2022; Klein, Anthony, and McKeever 2021). Metabolomics research conducted in malnourished pigs and humans with cachexia can serve as a foundational comparison for

emaciated equids, but species-specific knowledge through dietary intervention periods is warranted (Jiang et al. 2016; Freemark 2015; Yang et al. 2018). Despite the vast array of information available pertaining to the increasingly popular field of metabolomics, none are dedicated to the malnourished horse or the refeeding and rehabilitation process over the nutritional recovery period. Research pertaining to the equine metabolome throughout stages of malnutrition and the refeeding process could provide insight into specific nutritional requirements and allow owners to make dietary adjustments accordingly. Thus, this study focused on evaluating the metabolome of various malnourished horses during the refeeding process to determine metabolic differences between the starved and rehabilitated horse during the refeeding process.

Materials and Methods

Equine Enrollment

Ten, light breed horses aged 19 ± 3 years were enrolled into the study over a three-month period (Table 1). Horses for the study were obtained from three outlets: 1) in cooperation with the Tennessee Department of Agriculture (TDA) and the Tennessee State Veterinarian by owner surrender as a result of neglect cases, 2) purchased from public auction, and 3) private owner surrender. Enrollment criteria included: 1) BCS of 2 or less (Henneke et al. 1983), 2) light breed mares or geldings, and 3) apparent good health, aside from malnourishment, as defined by temperature, pulse and respiration rates within normal limits, and no evidence of coughing or nasal discharge at the time of enrollment. Due to the nature of horse acquisition and enrollment, previous diet history on each horse could not be acquired. All study procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee (Protocol #2811-1220).

Study Timeline and Sample Collection

The first ten days of the study were labeled as the critical care period (CCP). During this period, horses were housed and maintained at the University of Tennessee College of Veterinary Medicine (UTCVM; Knoxville, TN, USA) where their initial health status was evaluated by a

Table 1. Available metadata for equine subjects enrolled in the refeeding program.

Subject Identifier	Sex	Breed	Approximate Age (Years)
A	Mare	Quarter Horse	19
A2	Mare	Tennessee Walking Horse	22
B	Mare	Appendix	20
C	Mare	Quarter Horse	20
D	Mare	Tennessee Walking Horse	20
E	Mare	Quarter Horse/Arabian	18
F	Gelding	Appendix	16
G	Gelding	Appendix	23
H	Mare	Standardbred	15
I	Gelding	Quarter Horse	17

veterinarian and a Coggins test was performed. Blood samples were collected from the jugular vein on the day of intake and every other day thereafter. Weekly, horses' BCS were assessed by two individual, trained reviewers (Schneider et al. 2021). Vital signs including temperature, pulse, respiration, capillary refill time, and skin tent were assessed every 12 hours during the CCP to monitor horses for disease onset and overall health status in compliance with UTCVM policy. If horses displayed signs of potential infection, a respiratory swab was performed to check for Equine Herpes Virus (EHV), Strangles, and Influenza. If horses tested positive for any of these diseases, at-tending veterinarians worked in conjunction to provide care and treatment while maintaining refeeding protocols. Body weight (BW) was assessed daily using a commercially available equine scale (Optima, Rancho Cucamonga, CA).

The remainder of the study duration (days 10 – 165) was designated as the recovery period (RP). During the RP, horses were housed and maintained at either UTCVM, the University of Tennessee Veterinary Research and Education Center (VREC; Knoxville, TN, USA), or at Middle Tennessee Research and Education Center (MTREC; Spring Hill, TN, USA), based on housing availability. Horses were always moved as a cohort, and at least six days of acclimatization was allowed before the next sampling period. Before transportation, all horses tested negative for the infectious diseases.

Weekly BW measurements were taken throughout the duration of the study. Every two weeks, BCS was evaluated by two independent trained reviewers for each horse and jugular venipuncture blood samples were collected at each BCS increase. Fecal samples and respective fecal float and centrifugation tests were performed on Day 14 and horses were dewormed based on fecal test results. Horses were determined to progress to the next BCS when all reviewers agreed that the next BCS was reached, and a weight gain of 16 – 20 kg from the previous BCS was achieved (Heusner 1993; Council 2007). If horses reached a BCS of 4 before the end of the rehabilitation period, final measurements and samples were taken prior to study disenrollment. Plasma blood samples were maintained on ice until centrifugation at 3000 x g for 10 min. Serum blood samples were allowed to clot overnight and then centrifuged at the same speed and duration. All aliquots were stored at –80° C until analysis.

Diet Formulation

Upon enrollment, each horse was randomly assigned one of two diet groups for the duration of the study: control (C) diet which consisted of timothy hay and a commercially available vitamin mineral product (Purina Free Balance) where diets were formulated to meet DE requirements from forage alone; and senior (S) diet, in which 50% of DE requirements were provided from a commercially available complete feed, and the remaining 50% provided by timothy hay (Table 2). Diets were formulated to be isocaloric and meet daily National Research Council (NRC) nutrient requirements for mature horses weighing under 600 kg.

The refeeding diet protocol was established based on previously published methods (Stull, Hullinger, and Rodiek 2003; Weaver et al. 1998). During the CCP, from day of intake until Day 3, horses were fed 50% of daily digestible energy (DE) requirements split into six feedings. On Days 4 and 5, feeding amount increased to provide 75% of daily DE requirement over six feedings. On Days 6 to 10, horses were fed 100% of daily DE requirement over three different feedings.

Diets were formulated to provide adequate DE for 0.45 kg of BW gain per day. Initially, DE was increased to 125% of daily DE requirements based on weekly BW measurements. If horses did not gain weight at an appropriate rate for two consecutive BW measurements, DE was increased by 10%, to 135 or 145%, respectively.

Blood Chemistry Analysis

Plasma and serum chemistry quantitative values (Table 3) were determined via photometric absorbance values utilizing a COBAS C311 analyzer under guidelines determined by the manufacturer and utilizing approved reagent and standards (Roche, Switzerland).

Table 2. Nutrient analysis of forage of diet components.

Nutrient	Timothy Hay	Equine Senior	Free Balance Mineral
Digestible Energy (mCal/kg)	1.95	2.7	0
Protein (%)	9.88	15.5	1.55
Lysine (%)	0.34	0.7	N/A
Fat (%)	1.29	6.2	0.1
Acid Detergent Fiber (%)	39.50	22.54	N/A
Neutral Detergent Fiber (%)	62.23	38.89	N/A
Calcium (%)	0.21	0.77	13.52
Phosphate (%)	0.27	0.55	12.66
Potassium (%)	2.39	1.6	0.66

Table 3. Measured parameters for blood plasma and serum.

Analyte	Abbreviation	Units	Matrix
Lactate dehydrogenase	LDH	U/L	Plasma / serum
Alkaline phosphatase	ALP	U/L	Plasma / serum
Albumin	ALB	g/dL	Plasma / serum
Blood urea nitrogen	BUN	mg/dL	Plasma / serum
Gamma-glutamyl transferase	GGT	U/L	Plasma / serum
Aspartate amino transferase	AST	U/L	Plasma / serum
Glucose	GLUC	mg/dL	Plasma / serum
Phosphate	PHOS	mg/dL	Plasma / serum
Total protein	TP	g/dL	Plasma / serum
Alanine transaminase	ALT	U/L	Plasma / serum
Creatinine	CREAT	mg/dL	Plasma / serum
Triglycerides	TRIG	mg/dL	Plasma / serum
Creatine kinase	CK	U/L	Serum only
Non-esterified fatty acids	NEFA	mEq/L	Serum only
Serum calcium	SCA	mg/dL	Serum only

Metabolomics Extractions

The metabolomics extraction procedure was adapted from Rabinowitz and Kimball (Rabinowitz and Kimball 2007). Briefly, 100 μ L of plasma was mixed with 1.3 mL of chilled extraction solvent (4:4:2 acetonitrile:methanol:water with 0.1% formic acid) in an Eppendorf tube and allowed to extract for 20 minutes at -20°C. Samples were centrifuged and the supernatant was pooled into a separate glass vial. To the solution remaining in the Eppendorf tube, 200 μ L of extraction solvent was added, the mixture was vortexed, and allowed to extract for 20 minutes at -20°C. Samples were then centrifuged, and the supernatant was pooled into the glass vial with the supernatant from the first extraction. Pooled samples were dried under nitrogen and resuspended in 300 μ L of water prior to mass spectrometric analysis.

Ultrahigh Performance Liquid Chromatography Mass Spectrometry High Resolution Mass Spectrometry (UHPLC-HRMS)

The metabolomics analyses were conducted at the Biological and Small Molecule Mass Spectrometry Core at the University of Tennessee Knoxville (Knoxville, TN, USA). The sample extractions, chromatographic separations, and mass spectral analyses were performed according to an established method using UHPLC-HRMS (Bazurto et al. 2017). The collected metabolomes were stored at 4°C in an UltiMate 3000 RS autosampler (Dionex, Sunnyvale, CA, USA) until analyses were performed. All chromatographic solvents used were HPLC grade (Fisher Scientific, Hampton, NH, USA). Reverse phase separations were carried out using a Synergi 2.6 μ m Hydro RP column (100 mm x 2.1 mm, 100 Å; Phenomenex, Torrance, CA, USA) and an UltiMate 3000 pump (Dionex, Sunnyvale, CA, USA). The chromatography utilized a 25 minute binary gradient elution using a 97:3 water:methanol mixture with added 10mM tributylamine and 15 mM acetic acid (mobile phase A) and 100% methanol (mobile phase B). The flow was set to 0.200 mL/min, and the gradient was as follows: 20% B at 5 minutes, 55% B at 13 minutes, 95% B at 15.5 minutes, 0% B from 19 to 25 minutes. The separated metabolites were ionized via negative mode electrospray ionization prior to analysis on an Exactive Plus Orbitrap MS (Thermo Scientific, San Jose, CA, USA). The resolution of the mass spectrometer was set to 140,000 with an automatic gain control setting of 5e5 and injection time of 100 milliseconds. The sheath, auxiliary, and sweep

gases were set to 25, 8, and 3 arbitrary units, the spray voltage was 3.5 kV, and the capillary temperature was 320°C.

Data Analysis

Descriptive statistics for refeeding parameters were assessed using STATA SE (version 16.1, StataCorp, College Station, TX).

To evaluate the known metabolites, raw data collected by the instrument were converted to .mzML files via MSConvert (ProteoWizard). Features were then picked using an in-house library in EL-MAVEN based on exact mass (5 ppm) and retention time (± 0.5 minutes). Unidentified features were evaluated via Metaboanalyst 5.0 (Pang et al. 2021). Spectra in the form of .mzML files were compressed, uploaded to Metaboanalyst, and processed using the Auto-optimized feature in the LC-MS Spectra Processing module. As some batch effects were observed, features that were found to significantly vary (p value < 0.05) between batches based on an analysis of variance (ANOVA) were removed to lessen the chance of false statistical assumptions; this resulted in analysis of 26 known metabolites and 459 unidentified features. Heatmaps were generated using R with a custom script. Known and unidentified features were uploaded to Metaboanalyst for statistical analyses and visualization using the following parameters: features with missing values ($< 25\%$) replaced by one-fifth of the minimum positive value, normalized by sum, and log transformed. All other statistical analyses (e.g., partial least squares discriminant analyses or PLS-DAs) were performed using Metaboanalyst. Venn Diagrams were created using an in-house Python script.

Equine bloodwork was statistically evaluated in Metaboanalyst by performing 2-sample unpaired, equal group variance T-Tests between various groupings (CCP vs RP, diet, sex, and age group) using the same normalization and transformation procedure as metabolomics data. Box plots were generated using an in-house Python script.

Statistical conclusions based on p values were categorized as follows: p values of ≤ 0.01 , ≤ 0.05 , and ≤ 0.1 reflect statistical assumptions made with confidence intervals of 99%, 95%, and 90%, respectively.

Results

Equine Refeeding Outcomes

Originally, ten equids with a BCS of 2 or less were enrolled into the project; however, due to health circumstances, four horses were not able to complete the refeeding process. Specifically, the following situations occurred for each horse removed from the project (termed “Did not complete” in Table 4): (1) impaction colic resulting in euthanasia on Day 6 of the CCP; (2) positive respiratory swab for Equine Herpes Virus 1, wild type (EHV-1), *Streptococcus equi*, and influenza type A virus, resulting in euthanasia on Day 8 of the CCP; (3) positive respiratory swab for EHV-1, resulting in euthanasia on Day 1 within the RP; and (4) positive respiratory swab for influenza virus type A resulting in subsequent pneumonia, and due to lack of body weight gain was disenrolled on Day 73 of the RP. The remaining six horses (C diet $n = 3$ and S diet $n = 3$) were refed successfully to either a BCS of 3.5 or 4 (termed “Completed” in Table 4).

Initial weight at enrollment was 368 ± 58 kg (mean $\pm$ standard deviation; $n = 10$) and final weight after refeeding was 426 ± 54 kg ($n = 6$). Of the six horses that completed the project, the entire refeeding process from enrollment to successful disenrollment lasted 129 ± 28 days.

Analysis of Blood Chemistry Panels

Box plots were created to display differences in blood chemistry analyte levels between the CCP and RP (Figure 1). Results indicated statistical significance in analytes present in blood plasma samples (Supplementary Table S1). LDH, ALB, AST, and CREAT levels were all statistically increased during the RP compared to the CCP. Evaluation of blood serum corroborated a significant change in LDH, ALB, and CREAT (Supplementary Figure S1). Non-esterified fatty acids were also shown to significantly decrease in serum during the RP. Statistical differences

Table 4. Outcomes for equine enrolled in the refeeding program.

Subject Identifier	Program Outcome	Samples Taken	
		<i>CCP</i>	<i>RP</i>
A	Did not complete	5	NA
A2	Did not complete	7	NA
B	Did not complete	7	NA
C	Completed	7	4
D	Did not complete	7	1
E	Completed	7	4
F	Completed	7	3
G	Completed	7	3
H	Completed	7	3
I	Completed	7	2

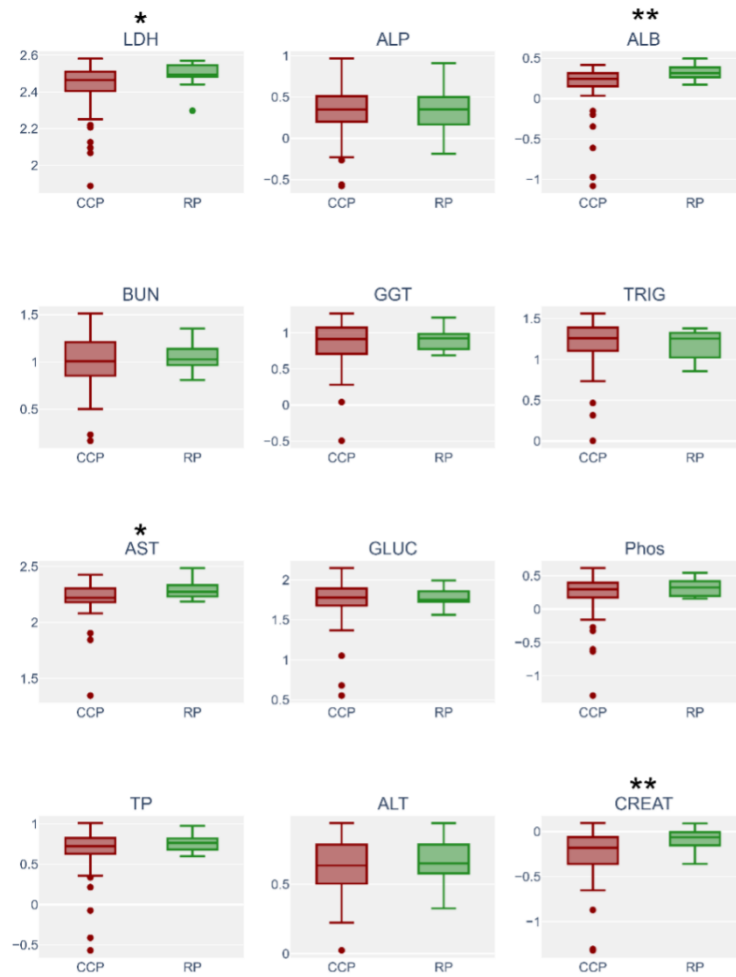


Figure 1. Box plots of blood plasma data conveying the differences between blood during the CCP (n = 68) and the RP (n = 15). Asterisks represent statistical significance (p value $\leq 0.1 = *$; p value $\leq 0.05 = **$). LDH: lactate dehydrogenase (p value 0.06), U/L; ALP: alkaline phosphatase, U/L; ALB: albumin (p value 0.04), g/dL; BUN: blood urea nitrogen, mg/dL; GGT: gamma-glutamyl transferase, U/L; mg/dL; TRIG: triglycerides, mg/dL; AST: aspartate amino transferase (p value 0.09), U/L; TP: total protein, g/dL; GLUC: glucose, mg/dL; Phos: phosphate, mg/dL; ALT: alanine transaminase, U/L; CREAT: creatinine (p value 0.04).

were also detected when comparing diet, sex, and age group in blood plasma and serum samples (Supplementary Tables S2–S7).

Fold Change Analysis of Metabolites Detected in Equine Subjects during the CCP and RPs

Fold changes (i.e., comparisons of metabolite abundance between sample types) were calculated for metabolites present in blood plasma. Of the metabolites detected, those classified as amino acids (amino acids, precursors, derivatives, and metabolism byproducts) showed the most perturbations (Figure 2). Overall, amino acid related metabolites such as glutamine, methionine, phenylalanine, alanine/sarcosine, and creatine showed statistically significant decreases during the RP compared to the CCP; however, *N*-acetylornithine showed a significant increase in abundance during the RP. Additionally, metabolites such as uridine, allantoin, and uric acid showed significant decreases. With the exception of *N*-acetylornithine, metabolites showing significant fold changes exhibited an overall decreased abundance during the RP compared to the CCP (Supplementary Table S8).

Differences of the Metabolic Profiles of Equine Subjects in the CCP vs. the RP

PLS-DA multivariate analyses allow complex data (i.e., metabolomics data) to be reduced to a simple three-dimensional scores plot, which clusters data based on variability between the samples (Worley, Halouska, and Powers 2013; Council 2007). Using these plots, one can generate hypotheses as to potential differences in metabolomic profiles between groupings, as well as investigate the metabolites that drive those differences. A PLS-DA was performed on the metabolomics data (known metabolites and unidentified features) to investigate potential differences in metabolomic profiles between equine in the CCP vs. the RP. Comparison of features unique to these groups resulted in a clear separation (Figure 3). As the known metabolites represent only a small portion of the data presented here, unidentified features were included in this analysis as these features provide insight into metabolic processes that have not yet been well described and contribute to a global view of all characteristic data points in a sample, serving as an important component of metabolic profile. Data from these analyses, combined with statistical fold change analysis, suggest that differences in the metabolome can be observed for equine subjects in the CCP vs. those in the RP undergoing the refeeding process.

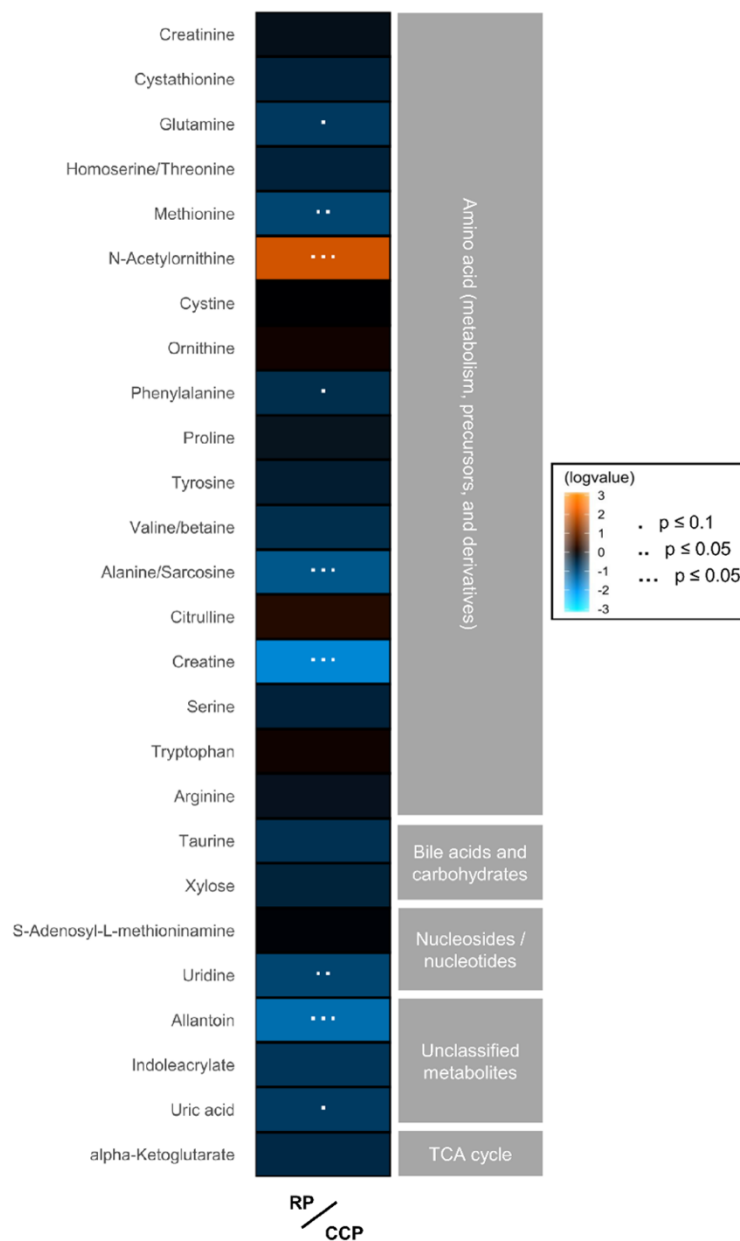


Figure 2. Heatmap showing increased or decreased abundance of detected metabolites organized by compound class between the RP ($n = 20$) and the CCP ($n = 68$; fold change of RP/CCP). Eight compounds exhibited significantly lower abundance while one compound showed significantly higher abundance during the RP.

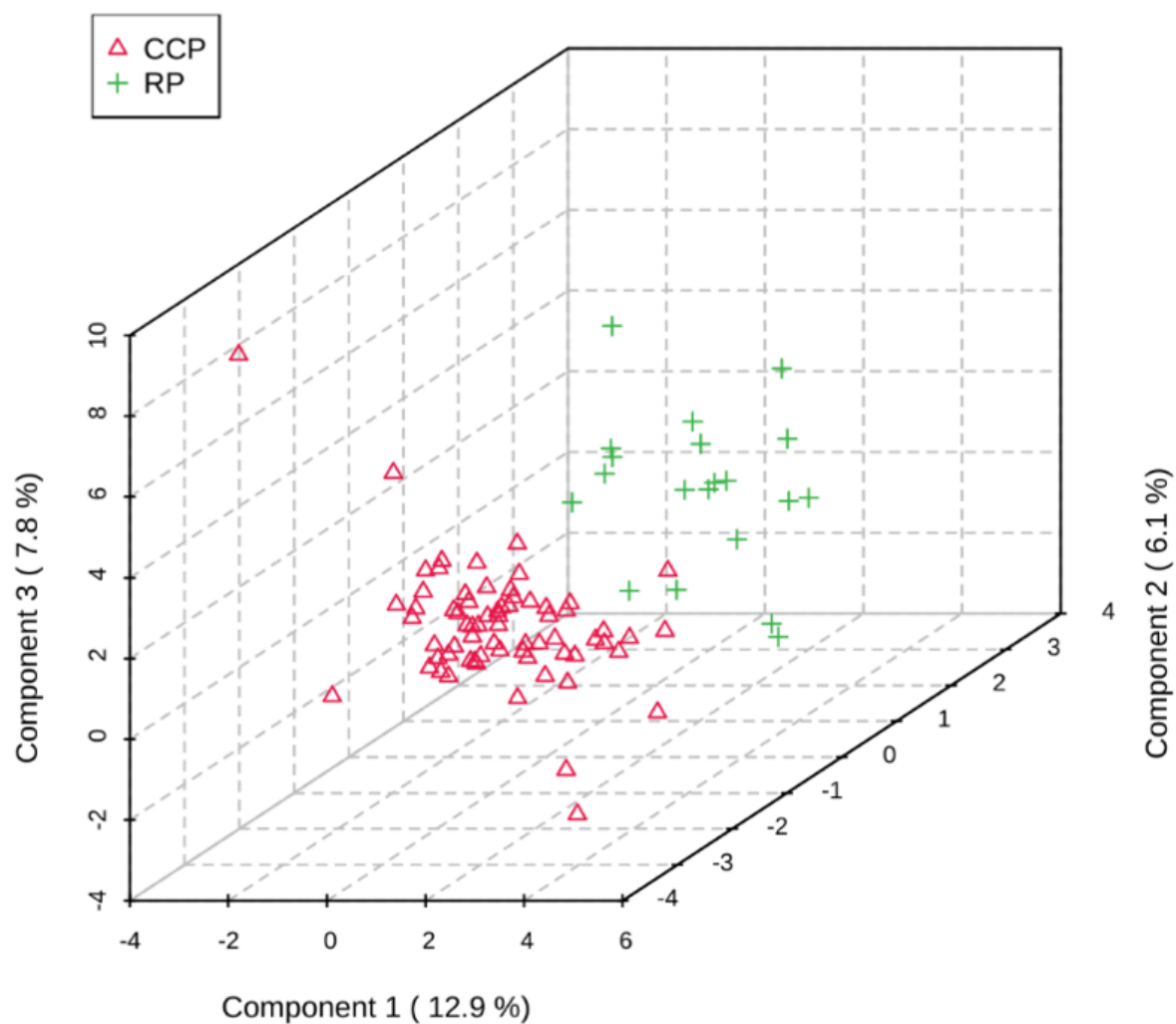


Figure 3. PLS-DA containing known metabolites and unidentified features detected during the CCP (n = 68) and RP (n = 20). The resulting plot shows separation between the groupings, indicating differences in the equine metabolomic profile during the CCP and the RP.

Effects of Subject-Specific Attributes on the Metabolome

Metabolic profiles (including known metabolites and unidentified features) were compared using the variety of metadata provided within the study: diet (C vs. S), sex (mare vs. gelding), and age group (below 20 years old vs. 20 years old and above). Analysis on breed specifications were not conducted due to low replicates (e.g., only one equine subject was of the Standardbred breed). PLS-DAs comparing diet, sex, and age group showed distinct groupings (Figure 4). Features were evaluated using a *t*-test and yielded 127, 157, and 40 statistically significant compounds (p value ≤ 0.1) for diet, sex, and age comparisons, respectively (Supplementary Tables S9–S11).

Components that drive separation in the PLS-DA are characterized by a higher variable importance projection (VIP) score (i.e., VIP scores greater than 1; Supplementary Tables S12–S14). A Venn Diagram was used (Figure 4) to gauge feature-based similarities and differences between subject-specific attributes. Overall, 33 features (including known metabolites and unidentified features) were common to all three groups evaluated, suggesting these features are common across the equine used in this study. Features that were unique to each group were much higher for sex and diet (22 and 28, respectively) than that of age group, which possessed eight unique features.

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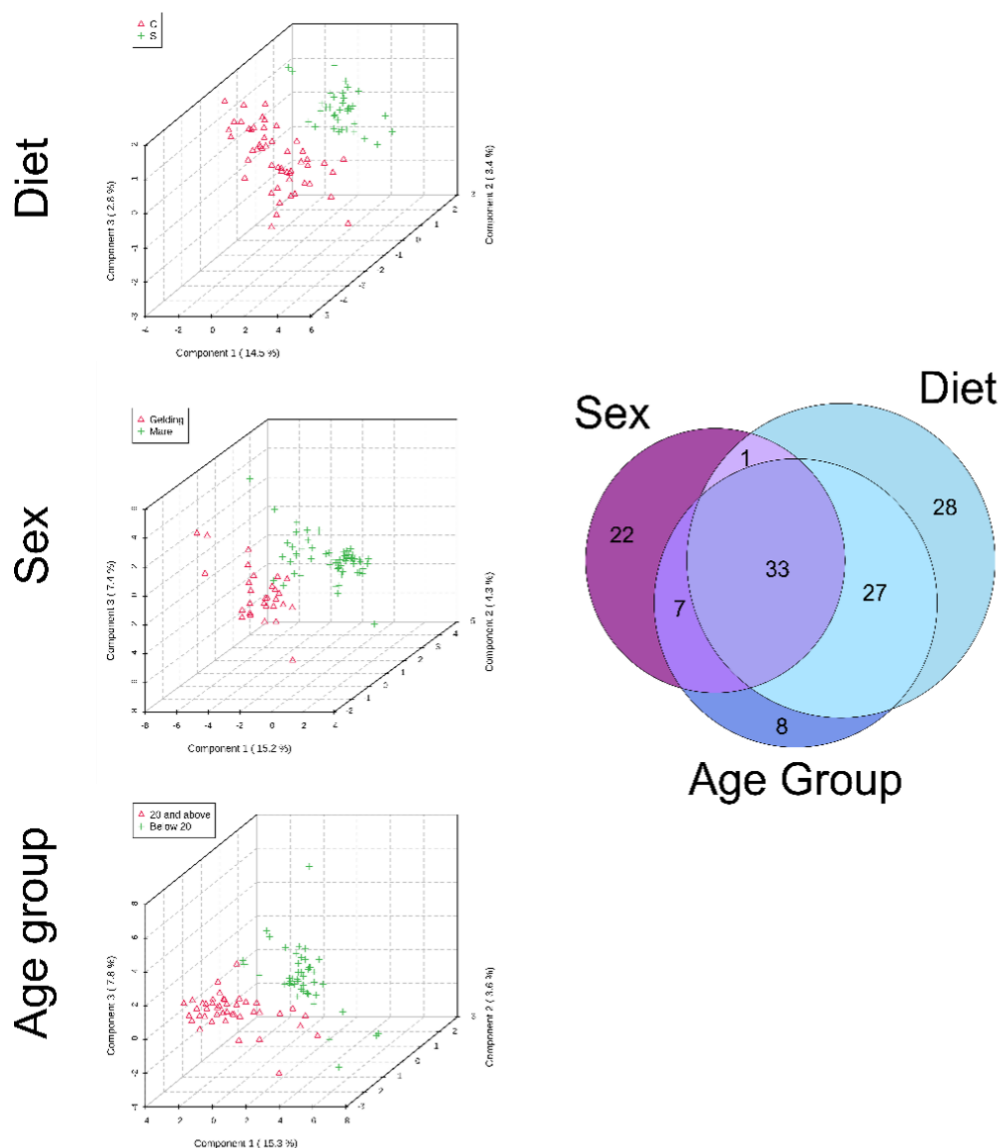


Figure 4. PLS-DAs (left) of known metabolites and unidentified features detected in the metabolomes of all horses that were enrolled in the refeeding program grouped by diet (C vs. S), sex (mare vs. gelding), and age group (below 20 and 20 and above). Sample sizes used for PLS-DAs were as follows C: n = 52; S: n = 36; mare: n = 59; gelding: n = 29; below 20: n = 45; 20 and above: n = 43. Venn Diagram (right) displaying similarities and differences between features in each grouping.

S14). A Venn Diagram was used (Figure 4) to gauge feature-based similarities and differences between subject-specific attributes. Overall, 33 features (including known metabolites and unidentified features) were common to all three groups evaluated, suggesting these features are common across the equine used in this study. Features that were unique to each group were much higher for sex and diet (22 and 28, respectively) than that of age group, which possessed eight unique features.

Discussion

Animal Origin and Source

Large animal nutrition studies create unique opportunities and challenges due to management, availability, and cost for such trials. Within our study, emaciated animal availability that met the enrollment criteria were difficult to source during the recruitment period. Extensive respiratory disease was prevalent in many potential animals that met the desired BCS (1 or 2). During the enrollment process an additional 12 horses were assessed for respiratory disease and all were positive EHV-1. Given the risk of transmission and morbidity rate for infectious respiratory diseases, alongside physiologic changes present during starvation, the pool of horses available to utilize for the project was limited (Pusterla et al., 2021).

Despite low sample numbers, power analysis prior to study approval and commencement indicated that animal numbers utilized in this study are adequate to detect statistical significance between treatment groups. Additionally, equine nutrition studies utilizing low animal numbers have been successfully completed and contribute meaningful information to the existing literature to advance clinical and management level decisions (Fleurbaey et al., 2001; O'Connor et al., 2008; Saastamoinen et al., 2021).

It is important to note that the backgrounds and histories of the horses enrolled in this study were largely unknown due to the variance in origin and ownership of the equine subjects. For this reason, it was not possible to consider factors that may influence the metabolome, such as diet history and

the presence of past or current parasites, comorbidities, and disease. Perhaps these factors should not be considered as no two subjects will present with identical histories and such diversity contributes to a better overall understanding of rehabilitating emaciated horses. Despite these unknown variables, statistical analyses conducted on the refeeding process indicate a distinct difference between the equine metabolome during the CCP vs. the RP. This study supports the necessity of the refeeding process for the malnourished horse via blood chemistry and metabolomics analyses, despite its history and individual attributes.

Blood Chemistry Analysis Suggests Improved Biological Function during the RP

Statistical analysis of each subject's blood chemistry during the refeeding process yielded significant changes in albumin, lactose dehydrogenase, aspartate amino transferase, creatinine, and non-esterified fatty acid levels between the CCP and RP. Albumin, a family of proteins primarily synthesized by the liver, was detected at lower levels during the CCP compared to the RP (Parraga, Carlson, and Thurmond 1995). Low albumin levels in equine were previously thought to be associated with liver disease and/or liver failure, but a retrospective study of literature data concluded that consistently low albumin levels (hypoalbuminemia) reported were not directly associated with severe liver disease (Parraga, Carlson, and Thurmond 1995). This finding does not dismiss the possibility that low albumin levels can be associated with improper hepatic function due to insufficient biological resources, such as those unobtainable during starvation.

Creatinine, a small molecule prominently present in plasma, was significantly altered between the CCP vs. RP. This nitrogenous molecule (Urayama et al. 2018; Hollis, Boston, and Corley 2007) used to evaluate renal function and overall muscle mass was found to be lower during the CCP than that of the RP (Toribio 2007). This compound is consistently produced in equine as a function of their muscle mass and body weight, dependent on proper renal function (Toribio 2007). This finding directly correlates to increasing BCS scores during the refeeding process; as the malnourished horse undergoes the refeeding process and their BCS score increases, a positive

correlation with increasing creatinine levels is observed, most likely due to a gain in muscle mass and body weight.

Levels of non-esterified fatty acids, compounds directly related to metabolic energy, were considerably lower during the RP. A lack of resources utilized for energy production (i.e., nutrient consumption) combined with consistent energy demands results in an overall negative energy balance (Doll et al. 2022). Increased levels of non-esterified fatty acids have been positively correlated with an overall negative energy balance in many fasted mammals, including equine, as body fat is broken down (into non-esterified fatty acids) and utilized as an energy resource as a last resort (Farhana and Lappin 2022; Zoppini et al. 2016). The study of starved horses supports this finding as higher levels of these compounds were observed during a state of malnourishment (i.e., the CCP) and decreased during the RP, suggesting that the energy balance was restored once the horse was provided with proper nutrients.

Lactate dehydrogenase and aspartate aminotransferase are two enzymes that serve essential roles in anaerobic and aerobic metabolic pathways, respectively, with important emphasis in hepatic, renal, and skeletal muscular metabolism (Klein et al. 2020; Zoppini et al. 2016). Interestingly, these enzymes serve as non-specific, clinical biomarkers of disease as their high-level presence is associated with cellular death (Farhana and Lappin 2022; Zoppini et al. 2016). In the present study, levels of these enzymes were statistically higher in equine during the RP than the CCP, which was an unanticipated observation as horses are not likely to experience as much cellular death during the RP compared to the CCP. Interestingly, increased levels of lactate dehydrogenase and aspartate aminotransferase have also been observed in a Japanese breed of equine that was fed a roughage-based diet; it was theorized that these enzymes were primarily higher due to diet (Ono et al. 2019). Despite statistical increases observed in the present study, the average levels of these enzymes were within reference ranges for equine in both the CCP and RP, suggesting an increase not necessarily indicative of disease (FIELDER 2015).

Blood chemistry panels are an efficient way to measure overall health and function of major organs, such as the liver and kidneys. However, these panels are limited in the amount of information attainable due to the sensitivity of methods and instrumentation. This problem of

sensitivity is solved when characterizing blood (i.e., plasma) via metabolomics methods, which provide a vast array of quantitative information on the complexities of numerous metabolic pathways. Though metabolomics methods only measure small molecules (unlike blood chemistries which can detect small molecules, proteins, and enzymes), the variety of metabolites detected and low concentrations at which these compounds can be measured provide more global information on the metabolic processes, without the need for separate protein or enzyme analysis. Therefore, blood chemistries were used herein as a supplement to the more globally informative metabolomics analysis.

Potentially Toxic Metabolites and Indicators of Oxidative Stress Were in Lower Abundance during the RP

Fold change analysis of metabolites detected during the RP compared to the CCP showed perturbations in metabolites with varying biological activity (Table 5). It is important to note that fold change analyses of global metabolomics data may show increases or decreases in overall abundance but cannot discern the ultimate metabolic fate of a particular metabolite. For example, if a metabolite is found in higher abundance, it is possible that this compound is produced by the biological system at a higher rate resulting in higher abundance; alternatively, this compound may be utilized by the system at a lower rate that also results in a higher concentration. To determine which of these scenarios is actually occurring, studies utilizing stable-isotope labeled metabolite tracers would be required to measure the fluxes of the metabolites of interest and ascertain the activity of pathways utilizing these molecules (Jang, Chen, and Rabinowitz 2018). Nonetheless, one can often infer whether metabolites are being actively metabolized by looking at changes in several or all molecules in pathways in the context of the system state instead of analyzing data for individual molecules.

During the study, it was observed that metabolites shown to exhibit toxicity when found in high concentrations were found in lower abundance during the RP. In particular, allantoin, phenylalanine, and methionine were found to be significantly decreased in horses with higher BCS scores. Allantoin and methionine are both small molecules that are often considered indicators of oxidative stress, a condition marked by increased levels of free radical species that

Table 5. Biological significance of metabolites observed to have significantly increased or decreased between the RP and the CCP.

Metabolite Class	Significantly Increased or Decreased (RP/CCP)	Metabolite	Biological Significance ¹
Amino acids (metabolism, precursors, derivatives)	Decreased	Glutamine	• Important non-essential amino acid • Synthesized by skeletal muscle
	Decreased	Methionine	• Sulfur-containing amino acid indicative of oxidative stress at high concentrations
	Increased	<i>N</i> -acetylornithine	• Primary metabolite commonly found in food sources (e.g., wheat) • Precursor to the urea cycle
	Decreased	Phenylalanine	• Essential amino acid that acts as a toxin at high concentrations • Used in the synthesis of proteins
	Decreased ²	Alanine	• Highly concentrated in muscle • Functions as a major energy source and regulator of glucose metabolism
		Sarcosine	• Often detected in muscle • Metabolic product of creatine
	Decreased	Creatine	• Generally found in skeletal muscle • Needs <i>methionine</i> as a catalytic precursor • Metabolizes to energy products, urea, or sarcosine
Nucleosides / nucleotides	Decreased	Uridine	• High levels associated with diet supplementation

Table 5 continued

Unclassified metabolites	Decreased	Uric acid	• Final product of purine metabolism • Metabolic precursor to <i>allantoin</i> • Potentially a toxic metabolite and is often excreted
	Decreased	Allantoin	• Metabolic byproduct of <i>uric acid</i> • Indicator of oxidative stress

¹ Biological significance gathered from the Human Metabolome database (<https://hmdb.ca/>; (Wishart et al., 2022)), the Livestock Metabolome database (<https://lmdb.ca/>; (Goldansaz et al., 2017)), and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg/>; (Kanehisa, 2019; Kanehisa et al., 2021; Kanehisa & Goto, 2000)).² These metabolites cannot be differentiated via the employed method; therefore, it is unknown whether they both decrease or singly decrease.

is often associated with disease and impaired nutritional status (Ghone et al. 2013). Specifically, methionine has been observed to be enhanced in subjects with severe acute malnutrition (Wishart et al. 2007; Foroutan et al. 2020; Ghone et al. 2013; Parenti et al. 2021). Because these potential biomarkers were found in lower abundance during the RP, it is theorized that the equine subjects are likely experiencing less oxidative stress due to these compounds acting as a defense against free radical species or their production at lower rates as their biological need to dissolve the radical species is lessened.

Similarly, phenylalanine appeared in higher abundance in equine during the CCP. Despite phenylalanine's proteinogenic properties, it serves as a toxic metabolite when found at chronically high concentrations. As this compound is an essential amino acid, an overall reduction in this compound's abundance during the RP may indicate an increase in health as this amino acid is potentially shunted to upregulate protein synthesis.

Metabolites Regulated by Skeletal Muscle Were Altered between the CCP and RP

Creatine was found to be significantly higher in abundance during the CCP. This finding supports a similar study conducted in juvenile pigs, which revealed that creatine levels were more abundant during a state of malnourishment (Jiang et al. 2016). This finding could be accredited to muscle wasting, a process in which muscle is broken down to be utilized for energy (Cohen, Nathan, and Goldberg 2015). It is theorized that muscle wasting may cause the release of creatine from the muscle cells into circulation, yielding higher plasma creatine levels in horses with lower body condition scores. It has been observed that creatine supplementation does not significantly increase plasma creatine level, furthering the notion that increased plasma creatine abundance in horses during the CCP is due to muscle wasting (Schuback, Essén-Gustavsson, and Persson 2010). As previously mentioned, methionine (a catalytic precursor of creatine), was also found in lower abundance in horses during the RP.

Glutamine is an important non-essential amino acid highly expressed in skeletal muscle, similar to that of creatine. Approximately 90% of all glutamine synthesized is via skeletal muscle; it is then transferred to the blood, where it represents the most abundant free amino acid (Wishart et al. 2007). Interestingly, this amino acid was present in lower abundance during the RP. Low levels of glutamine are often associated with disease, as consistent levels are required for proper function of many metabolic processes and organs (brain, lungs, gut, immune system, skeletal muscle, kidney, etc.). With this information and the observation that other toxic metabolites appear to decrease during the RP, it is more likely that glutamine levels are decreased in equine with higher BCS's due to high utilization by the system for other biological processes.

In addition to creatine, two metabolites commonly associated with skeletal muscle (alanine and sarcosine) were detected. The methods of detection utilized in this study were unable to differentiate these two isomeric compounds, yet a notable feature was detected either representing the isomers together or separately. In the event this feature is significantly changed due to a decrease in sarcosine (a product of creatine), a simple explanation of decreased abundance of precursors during the RP may be offered. Though, an alternative hypothesis is that muscle wasting and oxidative stress were overall less prevalent during the RP, resulting in decreased sarcosine abundance. Regardless, this cascade of metabolites was important to the discernment of the CCP and RPs. However, the metabolic feature associated with this finding is more likely due to the presence of alanine, as this amino acid is one of the more highly abundant amino acids found in plasma and is known to be found in higher concentrations than sarcosine (Bergero et al. 2005; Zhou et al. 2013). There are many theories as to why alanine was present in higher abundance during the CCP (including muscle wasting), but perhaps the most compelling is that of the shift in energy metabolism during starvation coordinated by metabolic crosstalk between skeletal muscle and the liver (Sarabhai and Roden 2019). Gluconeogenesis, glycogenolysis, glycolysis, and protein breakdown all retain the common factor alanine transaminase, an enzyme that produces alanine partly to prevent skeletal muscle from accumulating toxic nitrogenous products and to produce hepatic glucose (Sarabhai and Roden 2019). Thus, this offers an alternative to glucose homeostasis and energy metabolism (Sarabhai and Roden 2019). Alanine levels have also been observed to increase in equine during and after

exercise (i.e., when energy requirements change), which may mimic that of starvation (Bergero et al. 2005). Perhaps alanine was observed in greater abundance during the CCP due to high production amounts to satisfy the need of energy metabolism via hepatic pathways; yet, additional metabolites detected in this study provide evidence that hepatic function may be compromised, resulting in a build-up of this alternative energy product.

Significant Differences Were Observed in Metabolites Related to Liver and Kidney Function during the CCP and RPs

During the RP, uric acid, allantoin, and uridine were found in lower abundance while N-acetylornithine was found to be significantly increased. Uric acid is a purine derivative synthesized in the liver and, in excess amounts, is converted to allantoin, which is then filtered and excreted by the kidneys (Ishikawa, Aw, and Kaneko 2013). In mammals, allantoin reflects an endpoint of purine metabolism, unless it is shunted through the glyoxylate or glycine, serine, and threonine metabolism pathways (Kanehisa and Goto 2000; Kanehisa 2019; Kanehisa et al. 2021). The observation of altered levels of allantoin could be attributed to kidney function; starved, malnourished horses may experience kidney disfunction due to a lack of nutrients and resources, which prevent the ability of the kidneys to filter allantoin from the body. However, because uric acid is a precursor to allantoin and is not utilized in other metabolic processes (other than bile secretion), decreased levels of this compound indicate that it is being produced at a lower rate, yielding a lower rate of allantoin production (Kanehisa and Goto 2000; Kanehisa 2019; Kanehisa et al. 2021). Perhaps this is due to a lessened biological need for purine degradation, as these molecules are being directed to other pathways and are not present in excess.

Like uric acid and allantoin, uridine, a pyrimidine nucleotide important to RNA synthesis, is synthesized in the liver and excreted by the kidneys, if not catabolized (Zhang et al. 2020). Similar hypotheses exist for uridine as uric acid and allantoin. Lower levels are possibly detected during the RP as RNA is synthesized at a higher rate (i.e., less excess pyrimidine), which may indicate an increase in liver function. Yet, it's also likely that uridine is better excreted by the kidneys during this phase, resulting in lower plasma levels.

The only metabolite found to be in higher abundance during the RP was that of N-acetylmethionine, a small molecule involved in the biosynthesis of amino acids and a precursor to the urea cycle during arginine biosynthesis (Kanehisa and Goto 2000; Kanehisa 2019; Kanehisa et al. 2021). Endogenous arginine synthesis and the urea cycle primarily occur in the kidneys (Paddon-Jones, Borsheim, and Wolfe 2004). Because N-acetylmethionine was the only significantly altered metabolite involved in this cascade (arginine, citrulline, and methionine fold changes were not statistically different), increased production and subsequent utilization in the cycle is less likely than a build-up due to non-use. Isomers of N-acetylmethionine have been detected in high concentrations in various food sources, such as wheats and some fruits and vegetables. Thus, it is more likely that horses with higher BCSs that have undergone the refeeding process accumulate higher levels of N-acetylmethionine in plasma from their diet.

The Plasma Metabolome Illuminates Differences in Equine Subject-Specific Attributes

Statistically significant differences between specific metabolites and metabolic profiles were observed between the CCP and RP for ten equine subjects of various sex, age, breed, and origin. Differences were evident between designated diet, mares and geldings, as well as age group (below 20 years old and above 20 years old); yet, these differences were not evident in the analysis of the CCP vs. RP (e.g., sex-specific clusters were not evident in the CCP vs. RP discriminant analysis), indicating that these subject-specific attributes did not affect the conclusion that the metabolomic profiles of horses undergoing the refeeding process were statistically different.

As diet played an essential role in the current study, an analysis to determine potential differences in metabolomic profiles in equine assigned to a particular diet was conducted. Various statistical analyses suggest perturbations in metabolism with respect to diet. In the event of a negative energy balance (e.g., in the case of malnourishment), particular care is placed on diet such that the subject can consume and digest nutrients without inducing refeeding syndrome or overfeeding (i.e., the process of introducing nutrients in excess such that metabolic homeostasis cannot be maintained (Klein, Stanek, and Wiles 1998). Overfeeding may result in further metabolic damage, particularly in the liver and kidneys, both major organs which have

shown to be impaired during the CCP in the current study. Thus, diets for malnourished equine must be carefully tailored to slowly reintroduce nutrients in a manner such that further metabolic damage is not inflicted. Though an in-depth analysis of the effects of a particular diet or set of nutrients on equine outcome was not within the scope of this study, it is worth noting the differences observed in the global metabolome in this study; further investigation is required to determine the detailed effects of diet and nutrients that cause this separation.

With advances in equine feed that cater to aging horses, the average lifespan of the equid is increasing, which has prompted a surge in research devoted to older horses (i.e., horses 20 years old and above) (McKeever 2003). For this reason, an analysis to evaluate the metabolome of younger vs. older horses in the present study was conducted and resulted in clear differences between the two groups. The amount of metabolites that drove this distinction was not as prominent as other comparisons (i.e., diet and sex), though various statistical tests support perturbations based on age group. It is theorized that many metabolic factors may play a role in this age-based separation, including alterations in muscle fiber type and renal function (McKeever 2003). Similar to effects from diet, further studies are warranted to establish clear relationships between the aging horse and metabolism.

It is important to note that the analysis of the metabolome provides a biochemical snapshot of the metabolic state for the system, which is highly complex. For this reason, it is not surprising that attributes such as diet, sex, and age affect metabolic profiles. Moreover, these attributes contribute diversity to the study of the impacts of refeeding to gain a better understanding of caring for malnourished equine.

Conclusions

Comparison of the equine metabolome during a state of malnourishment versus rehabilitation illuminated significant differences that provide essential biological insight to the process of refeeding the emaciated horse. After commencement of the refeeding process (i.e., during the RP), a decrease in potentially toxic metabolites was observed, metabolic alterations suggesting increased liver and kidney function were shown, and metabolite changes related to muscle gain/repair and changing energy demands were evident. Blood chemistry panels also showed

statistical differences between analyte abundance between the CCP (i.e., state of malnourishment) and the RP, further indicating improper liver and kidney function during the CCP. The combination of the blood chemistry and metabolomics analyses on the equine subjects in this study provided essential insight to the biological processes that are influenced when rehabilitating the malnourished horse. Despite differences observed between the metabolome with respect to subject-specific attributes (i.e., diet, sex, and age group), a clear discernment between the metabolome unique to the CCP and RP were observed, providing evidence that the refeeding process has a profound impact on equine rehabilitation.

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CHAPTER III
EXTENSION EDUCATION: VARYING THE APPROACH TO
EDUCATION, ASSESSMENT, AND TRAINING

Introduction/Extension Overview

In 1862 the Agricultural Colleges Act, more commonly known as the Morrill Act was adopted. This piece of legislation provided funding, land acreage, and resources to implement higher education institutions geared towards “the common man.” Such institutions had the goal of providing practical skills and knowledge to those individuals who sought education and careers in the fields of agriculture, military sciences, and mechanics (Duemer, 2007). This led to the creation of Extension components of land-grant institutions that aimed to equip students with the knowledge and skills to solve everyday problems (McDowell, 2001). Each land-grant institution and therefore each state has a slightly different extension model. Agriculture and Natural Resources (ANR), 4-H Youth Development (4-H), Community Economic Development (CED), and Family and Consumer Sciences (FCS) are the four sectors that make up the University of Tennessee Extension model. Each of Tennessee’s ninety-five counties contains an Extension office with agents representing each of the aforementioned sectors. This model serves youth by creating personal development opportunities for youth across a variety of project areas. However, differing agent background and knowledge areas pose challenges to implementing successful programming in all topic areas for each county. Thus, in some circumstances, volunteers may be needed to aid in diversifying the available educational opportunities for youth.

Education Strategies

Cooperative Extension Services provide educational programming for a multitude of audiences across adult and youth demographics. Programs aim to increase the knowledge and skill sets of agricultural producers, the general public, and youth by taking science-based information and delivering it in a way that is easily understood and applied by target demographics. County agents work alongside state-level specialists and community volunteers to implement topic-specific educational programming in their counties for youth and adults alike.

Online Learning

Continuing advancements in technology along with lingering effects of COVID-19 have resulted in the increased popularity and use of online learning over the past several years (Huang, 2020). Overall post-secondary education enrollment has seen some decline while distance learning courses have seen an increase in enrollment (Allen & Seaman, 2013). With this drastic change in the dynamics of public and higher education comes the challenge of finding and executing effective techniques in regard to online instruction (Adedoyin & Soykan, 2020). Commonly, online learning methods are categorized as either synchronous or asynchronous (Hrastinski, 2008). With an asynchronous learning model, educators utilize tools including email and online discussion boards to allow students to interact with one another and the instructor while completing assignments without being online simultaneously (Hrastinski, 2008). On the other hand, a synchronous learning model utilizes online conferencing software such as Zoom to allow students to attend class remotely but without sacrificing the social interaction of an in-person learning atmosphere (Hrastinski, 2008). When implemented properly, certain techniques can prove very beneficial to the effectiveness and success of online learning platforms. In order for an online course to be effective, it must be user friendly (Cantrell et al., 2008). Designing the site to suite computer users of all skill levels, implementing a variety of learning tools (discussion boards, online quizzes, chat rooms, etc.), providing teaching personnel that respond to student questions in a timely fashion, and clearly outlining course expectations are several key factors that contribute to the success of an online learning platform (Cantrell et al., 2008).

In a study conducted by the School of Business at Madonna University, students were subjected to either an online and asynchronous course model or a fully face-to-face model where both courses were taught the same material by the same instructor (Neuhauser, 2002). It was reported that overall learner performance is not negatively affected when using an online learning model when compared to a face-to-face model (Neuhauser, 2002). In fact, when learners participating in this particular study were asked to identify their preference in learning model, they indicated that they found the online course to be equally or more effective than a face-to-face structured course (Neuhauser, 2002). It has also been indicated that teachers and students alike are satisfied with the overall success and effectiveness of strictly online learning models (Huang, 2020).

However, they also explained that 90% of participants expressed the inability to communicate with one another face-to-face was the biggest downside of a solely distanced learning model (Huang, 2020).

Face-to-Face Learning

Historically, face-to-face instruction has been the predominant teaching method for all facets of education despite the recent increase in distance learner enrollment (Allen & Seaman, 2013). Traditional instruction is commonly implemented in a synchronous, physical format and utilizes techniques such as instructor-led classrooms and lectures, hands-on labs and workshops, and field trips (Singh, 2021). As with any form of education, certain tactics and techniques should be implemented to ensure learner retention and success across all age demographics. In fact, poor academic performance from most students can be directly linked to ineffective teaching methods implemented by instructors (Adunola, 2011). Common face-to-face instruction techniques include lectures, group discussions, case studies, brainstorming sessions, presentations, workshops, etc. (Sajjad, 2010). Surprisingly, when asked to rate various strategies of traditional education, students indicated that lectures were the most effective and accredited this to technique's ability for instructors to deliver adequate information, the opportunity for learners to be attentive and take notes while learning through auditory engagement, and the ability for learners to ask for clarification or additional information if needed (Sajjad, 2010). On a 1-5 Likert scale, 40% of students rated lectures at 5, which is more than any other educational delivery method (Sajjad, 2010). The group discussion approach most closely followed lecture in perceived effectiveness by students with 36% of students rating this method at a 5 (Sajjad, 2010). It was indicated that this is due to the opportunity for social interaction with one another and their ability to share their personal outlooks and opinions with their peers in regard to course material and subject matter (Sajjad, 2010). On the contrary, a separate study found that student test scores were highest when engaging in a student-teacher interactive approach to learning rather than a teacher-centered approach such as lecture style teaching (Ganyaupfu, 2013). However, this study generated similar results in regard to student-centered educational methods where this technique yielded the second highest mean assessment score (Ganyaupfu, 2013).

Blended Learning Models

After discussing the techniques, strategies, and effectiveness of both face-to-face and distance instruction, it is then important to pose the question of whether one educational approach is superior to the other. Overall, it has been suggested in the literature that students have provided a slightly higher preference for face-to-face instruction when compared to a distance learning format but yielded little to no difference in satisfaction ratings (Allen et al., 2002; Carrol & Burke, 2010). However, opinions can differ based on a variety of factors and personal preferences such as perceptions of technology, personality, attitude, and skill level (Allen et al., 2002). As a result, vast differences in learning requirements and preferences, educational institutions across the globe have moved toward incorporating elements of both in-person and online distance instruction into more adaptive educational formats that came to be known as blended learning models (Singh, 2021). These hybrid models are often comprised of a mixture of self-paced online modules, live webinars or virtual class sessions, and scheduled face-to-face meetings (Singh, 2021). Hybrid learning models provide benefits of both traditional and online learning formats by allowing individuals to attend a classroom-based training while simultaneously allowing others to participate virtually, thus, extending the educational opportunity to individuals that would otherwise be unable to participate due to the time or location restraints of a strictly in-person approach (Singh & Reed, 2001). Blended models also allow for optimized development time and cost associated with the production of educational programs. For example, fully immersive, asynchronous online programs can become expensive to create due to software requirements and solely face-to-face options pose the aforementioned time constraints (Singh & Reed, 2001). An overall performance increase from learners has been reflected when engaging in a well-executed blended learning model compared to both face-to-face and online learning platforms (Hughes, 2007). A significantly lower failure rate was observed along with an increased rate of submitted assignments, thus suggesting that when utilized properly, blended learning formats can prove very beneficial to learner retention (Hughes, 2007).

When implementing educational programming through Extension, in-person, online, and blended models are utilized. However, little research has been done to evaluate and compare the

frequency and audience preferences of these models in an Extension setting. Additionally, there is a lack of research in the literature that evaluates the overall effectiveness of these models in the realm of Extension.

Extension/Agriculture Education

Importance/Overview

As previously noted, the mission of land grant institutions is rooted in providing solutions to every day issues in regard to agriculture and natural resource production, management, and sustainability (Grant et al., 2000). The first step to accomplishing this mission is effectively staffing institutions with personnel that are well-versed and properly educated in these content areas. Thus, agriculture education plays a vital role in the success of university extension programs. Traditionally, agriculture education takes the form of a three-circle model consisting of classroom instruction, experiential learning via supervised experiences, and leadership activities (Dailey et al., 2001). When implemented in a properly designed program, agriculture education as a three-circle model provides learners with not only content knowledge but also hands-on experience and soft skills that will prove beneficial in any agricultural field of work including that in a university extension setting (Dailey et al., 2001).

Extension Agent Background

Within the University of Tennessee Extension system, all 95 counties in the state employ agents that serve as representatives of the university to carry out various adult and youth programs within their respective counties. Each county extension agent is categorized into one of three program areas (ANR, 4-H, or FCS) with some agents having split appointments between multiple program areas. Adult education and programming geared toward agriculture and natural resources falls under the responsibility of ANR agents whereas youth programming, 4-H clubs, and school outreach is overseen by 4-H agents. In many cases, ANR and 4-H agents will work hand-in-hand to conduct certain programs based on the knowledge and skills possessed by each

agent. For example, a county 4-H agent may lack knowledge and background in livestock production and will reach out to the county ANR agent to help coach a judging team. Despite this system's overall success, it is common for this vast array of difference in knowledge between county extension agents to leave some counties lacking in certain topic areas (i.e., a county lacks a successful 4-H horse program due to county agents lacking equine focused knowledge or skills). Additionally, the county ANR agent may lack a background in animal agriculture altogether. This creates an even greater knowledge gap in county Extension personnel especially when partnered with a 4-H agent that also lack knowledge in animal-related topics. Regardless of knowledge, skills, and background, agents are often required to implement programs outside of their given specialty area. This practice often creates knowledge gaps in individuals across different counties. Often, agents have appointments with split responsibilities between ANR and 4-H programming which further exacerbates the ability to create effective educational opportunities in all topic areas. Furthermore, it is imperative that county extension agents continue to learn and develop their skills while embedded in the occupation. This is especially true regarding topic areas, content, and programming of which they consider unfamiliar. By obtaining knowledge of additional subject matter, county extension agents can expand their programs and create additional opportunities for youth and adults alike.

Often, county Extension agents rely on volunteers from the community to aid in conducting youth programming. This is especially true for those agents responsible for overseeing programs outside of their knowledge areas. For example, a county agent may rely on a knowledgeable equine enthusiast from their community to coach a horse judging team due to the agent's lack of equine-specific knowledge. In counties where this is the case, it is vital for those agents to recruit quality volunteers to lead youth clubs and programs in their county.

Previous Research Efforts on Educating Extension Personnel

The University of Tennessee Extension service along with other land-grant based extension services across the world implement successful programming to teach and train youth and adults across a vast variety of subject areas. With the growth and expansion of successful programming

come the need to train educators on successful delivery and utilization of said programs (Richards et al., 2012). This is especially true for county extension agents who are required to delivery programming beyond the scope of their specialty areas.

The overall effectiveness of Virginia's New Extension Agent Training (NEAT) program (Brown et al., 2008) was evaluated based the perceived importance and effectiveness of 70 Virginia Cooperative Extension employees who participated in the NEAT program regarding several competencies covered during the training (Brown et al., 2008). Overall, the NEAT program was vital when onboarding new agents and was also important in furthering the success of existing personnel (Brown et al., 2008). Results of this study indicated that the highest effectiveness ratings were given to the communication, educational processes, and program planning categories, furthering the idea that extension employees find value and benefit in annual training to better grow and expand the varying programs they oversee (Brown et al., 2008). Barriers and effective educational strategies were determined in order to develop the professional competencies of extension agents in North Carolina (Lakai et al., 2012). Respondents indicated that the three biggest barriers they faced on the job were increased work load, lack of time, and lack of funding (Lakai et al., 2012). However, lack of training opportunities and ineffective training delivery methods were also experienced to a somewhat large extend (Lakai et al., 2012). This is indicative that extension agents are not only open to engaging in training opportunities but are actively seeking out effective trainings to attend in attempt to greater their knowledge and skills, thus growing and expanding their facilitated programming. On the other hand, participants were asked to indicate the training strategies and formats that they perceived to be the most effective. Overall, responses reflected that the most preferred delivery method was face-to-face small group training workshops followed by a combination of two or more delivery methods and face-to-face large group training workshops (Lakai et al., 2012). It has also been determined that extension agents as a whole possess a large variety of preferred learning styles and modalities that contribute to their perceived success of a training session (Pigg et al., 1980).

Adopting the findings of the aforementioned research on the effectiveness, barriers, and strategies associated with educating extension agents and specialists could prove useful when

designing and implementing new agent training programs as well as when adapting existing ones to be more effective. It is crucial to consider the array of various learning styles, preferences in training formats, and personality types when beginning to design any educational program. By designing a program that appeals to all types of learning modalities, adopts multiple formats in the same training, and allows for adaptability, extension agents and other professional staff can receive valuable knowledge and skills that can be taken back to their respective county or region and used to increase retention and success within their programming. This idea is prominent when considering those agents who facilitate educational programming outside of their predominant knowledge area.

Measuring Performance

When administering educational programming, it is necessary to gauge the effectiveness and success of the program to determine its overall success. When it comes to accomplishing this goal and measuring success, it is common for outcome indicators to be utilized. Outcome indicators are quite complex and a true definition has not been agreed upon (Shavelson et al., 1990). However, they are often described as statistics that are used to quantify complex scenarios that could be subjective to human judgement (Shavelson et al., 1990). Commonly, tests and exams are used to measure student success (Scheerens et al., 2011). This includes tests from textbooks, state curricula, national curricula, teacher-designed curricula, and multi-faceted competencies (Scheerens et al., 2011). When using testing as a tool for knowledge evaluation, several things must be taken into consideration. Firstly, the format in which responses are recorded (multiple choice, open-ended, etc.) play an important role in administering tests (Schuwirth & Van Der Vleuten, 2004). It is recommended that open-ended questions be used to only to evaluate that which cannot be accurately recorded using multiple choice questions (Schuwirth & Van Der Vleuten, 2004). Additionally, the format of the stimulus or how the question on the test is constructed is more important than response formats when developing effective tests (Schuwirth & Van Der Vleuten, 2004). Furthermore, the stimulus format relates to the content of the question itself and whether knowledge alone is being evaluated or if the test taker is asked to use their knowledge in an applied or contextual scenario (Schuwirth & Van Der

Vleuten, 2004). Very little research has been observed regarding the use of contest results as a means of knowledge evaluation.

The 4-H Horse Hippology contest aims to prepare youth for success in the equine industry and test their knowledge against their peers in the form of competition. This contest has presence at the regional, state, and national level. The Tennessee Regional 4-H Horse Hippology contest is a multi-faceted youth competition where participants complete a written exam, slides phase, and judging phase to test their overall knowledge of horses and the equine industry. Based on their performance, participants then have the opportunity to advance to the state-level contest where they will compete in a similar contest with the addition of a stations phase. The Eastern National 4-H Hippology contest is only open competitors in the senior age division. This contest is structured similar to the Tennessee state contest. However, during the stations phase, competitors must complete the stations on their own as opposed to working as a team.

Train-the-Trainer Models

An important aspect of creating successful professionals is providing them with a good mentor. It has been stated that good mentoring is critical to success, especially in the world of education and academia (Sood et al., 2016). One strategy for establish good quality mentors and giving equipping them with the skills they need to mentor others is a Train-the-Trainer (TTT) approach. Train-the-Trainer models are educational systems often used by institutions containing subject matter experts to provide education, curriculum, instructional guidelines, and teaching methods and materials to other individuals in order to equip them to train on a particular subject (Orfaly et al., 2005). Commonly, these methods have been used to create effective teaching professionals across numerous industries including agriculture, health, professional development, medicine, and academia (Bess et al., 2003; Carruth et al., 2010; Orfaly et al., 2005; Pancucci, 2007; Sood et al., 2016).

Train-the-Trainer approaches have proven to be successful in the realm of public health preparedness according to a study done in Maine (Orfaly et al., 2005). After 1 year of

implementation, results indicated an overall success in this program, but there was no definitive structures or components that ensure this success (Orfaly et al., 2005). Additionally, only 20% of participants utilized the training they received within 6 months of completion due to lack of time and resources (Orfaly et al., 2005). Thus, it is vital that institutions and organizations seeking to implement a TTT program remain flexible in regard to the overall structure and format of their TTT model to cater it to their specific needs (Orfaly et al., 2005).

Additionally, TTT models have also seen success in professional development outlets and has proved to improve preparedness for teachers (Pancucci, 2007). Perceived effectiveness was evaluated of a TTT model that was utilized to train 83 elementary school teachers on the subject of information technology (Pancucci, 2007). Participating teachers engaged in four half-day trainings along with one after-school session and were asked to provide their perceived effectiveness of the training (Pancucci, 2007). Results indicated that teachers saw benefit in this TTT professional development training and gained confidence in their ability as Information Technology Coaches, proving that TTT models can be beneficial when incorporated into professional development activities in education (Pancucci, 2007). However, it was also determined that despite the success of this TTT program, it is vital to include a variety of additional training methods to successfully change teachers' practice (Pancucci, 2007).

In an academic setting, particularly higher education, TTT approaches are seen often when developing young and aspiring faculty members (Sood et al., 2016). In fact, seasoned faculty that also possess the skills of a great mentor are in short supply, thus the need for a TTT program that strives to build upon the mentorship skills of faculty that primarily engage in research (Sood et al., 2016). It has been suggested that two-pronged framework consisting of individual competencies and institutional climate for mentoring be used when developing and administering a TTT program for faculty mentors (Sood et al., 2016).

In the realm of agriculture and extension education, the use of TTT programs has seen much less attention in the literature when compared to applications of similar approaches in other disciplines. However, a study was done on high school agriculture education students to

determine if a TTT approach had an effect on knowledge gain and anticipatory action when compared to those who did not receive the training (Carruth et al., 2010). Results of this study indicate that individuals exposed to the training program reflected an increase in anticipatory action, but did not see an increase in overall knowledge acquisition (Carruth et al., 2010). Additionally, students indicated that they enjoy learning from their peers and those individuals that participated in the TTT program indicated that they felt more confident in their ability to teach their peers (Carruth et al., 2010).

Implications and Future Directions

Keeping these things in mind, it has been identified that there is a prominent lack of research in the areas of using youth educational contests as a tool for knowledge evaluation and the use of a train-the-trainer program within Extension and 4-H programs. Specifically, equine Extension programs lack efficient, metric-driven assessments of knowledge geared toward youth or adult audiences. Additionally, applied training programs have proven useful for county extension agents or other agriculture educators and has the potential to have the same effect in terms of equipping them to effectively deliver equine focused knowledge and skills to youth (Richards et al., 2012). Therefore, one goal of this study was to evaluate the knowledge base of youth using the Tennessee 4-H Regional Hippology contest and the Eastern National 4-H Hippology contest as a metric based assessment. Additionally, this project sought to create and evaluate an applied train-the-trainer program and associated curriculum for Tennessee Extension agents centered around the youth knowledge gaps identified by the aforementioned contests.

CHAPTER IV
USING YOUTH HIPPOLOGY CONTEST RESULTS AS A NEEDS
ASSESSMENT AS A FRAMEWORK FOR A TRAIN THE TRAINER
PROGRAM

Abstract

Extension agents and youth educators differ in areas of expertise, leaving some counties with minimal ability to provide 4-H horse project members with sufficient content knowledge while agents in other counties are more well versed in equine-specific areas. The objective of this project was to conduct a needs assessment using the Tennessee regional and Eastern National 4-H Hippology contests and implement a training program and curriculum to improve upon identified areas of deficiency. Youth (n=224) competed in the 2021 regional 4-H Hippology contest across the Eastern (n=59), Central (n=93), and Western (n=72) regions. Youth competed within their age divisions, where junior (grades 4 and 5, k=60), junior high (grades 6 to 8, k=100), and senior (grades 9 to 12, k=100) divisions competed in written exam and slide identification phases. Scores from all regional contests were collected and assessed to create a baseline for current equine content knowledge of 4-H members. Based on previously established equine project area learning outcomes in place since 2018, questions were placed into categories (colors, behavior, reproduction, ownership, selection (S), breeds (B), facilities, hoof care, tack (T), health (H), nutrition (Nu), waste management, exercise, and genetics) and areas of deficiency were identified within each age division based on percentage of questions answered incorrectly. Statistical analysis was performed using the frequency procedure in SAS v9.4 (Cary, NC). Nutrition (Nu) questions were the highest percentage missed by both senior and junior high youth (k=7.8, 51.94%; k=7.5, 44.22%, respectively) whereas junior youth missed T questions most frequently (k=4.9, 54.14%). Of the 5 topic areas of deficiency, S questions were the lowest percentage missed by both senior and junior high youth (k=5.9, 36.81%; k=7.1, 39.53%, respectively) whereas junior youth missed H questions least frequently (k=4, 39.87%). Breed related questions were consistently missed by youth across all age divisions as reflected by juniors missing 43.7% (k=3.1), junior high youth missing 41.54% (k=2.9), and seniors missing 40.28% (k=1.2). Upon implementation, it was found that training status had no significant on scores from year to year. However, when data were analyzed, significant effects were found when comparing across question category ($p<0.0001$), age group ($p<0.0001$), and year ($p<0.0001$).

Introduction

The University of Tennessee Extension service implements successful youth and adult educational programming to teach and train across a variety of agricultural subject areas. As these programs continue to grow in size and scope, the need arises to train educators on successful delivery and utilization of these programs (Richards et al., 2012). Currently, there is a statewide need for additional species-focused curriculum in Tennessee 4-H programs.

Extension agents across Tennessee differ in areas of expertise, leaving some counties with minimal ability to provide 4-H members with sufficient content knowledge on equine nutrition, health, and management, while agents in other counties are more well versed in these topic areas. Further, the impact of this knowledge gap on youth enrolled in 4-H programs and the ability to learn, retain, and apply equine management concepts is unknown. Specifically, within the 4-H Horse Project both in Tennessee and across the United States, project members are able to compete in the 4-H Horse Hippology Contest. This contest is designed to test the knowledge and application skills of horse project members. Typically, participants compete in teams of 4, grouped by age division (Junior, Junior High, and Senior) across several different phases of the contest. The overall goal and scope of these contests are the same. However, the structure and phase compositions differ slightly based on the level of the contest (regional, state, and national). For example, Tennessee regional contests consists of written exam, slides, and judging phases whereas the Tennessee State contest and Eastern National contest are comprised of written exam, slides, judging, and stations phases.

Commonly, tests and exams are used to measure student success (Scheerens et al., 2011). Additionally, poor academic performance can be directly related to teaching methods implemented by instructors (Adunola, 2011). However, there has been limited research on using youth educational contests as a means of measuring performance metrics. Therefore, this program utilized the aforementioned hippology contests as a tool for evaluation of student knowledge and took a “Train the Trainer” approach to improving equine content knowledge of 4-H members across the state of Tennessee through knowledge empowerment of ANR and 4-H agents.

Extension currently utilizes a train the trainer approach through the annual Joint Animal Science in-service training and monthly hot topics webinars. However, formalized curriculum and assessments regarding end-user knowledge gain, implementation, and application is not as common, and lacking within many livestock project areas. Additionally, the literature has shown that the implementation of Train-the-Trainer programs has yielded positive student knowledge gain (Bess et al., 2003; Carruth et al., 2010; Orfaly et al., 2005; Pancucci, 2007; Sood et al., 2016). This project collected and analyzed scores from regional (Eastern, Central, Western) Tennessee 4-H Horse Hippology contests, determined topic areas in which youth were lacking knowledge, developed a training program and associated curriculum for county extension agents, and re-evaluated scores from the same contest in the year following the implementation of the aforementioned training and curriculum. It has been shown that student preferences differ among learners in regard to online and in-person platforms, but both formats can provide successful learning outcomes (Sajjad, 2010; Neuhauser, 2002).

The objective of this study was to assess how different educational module deliveries can impact end-user knowledge and application by creating a training program and associated curriculum for Extension Agents using a train-the-trainer approach. We hypothesized that youth hippology contest scores would improve after the implementation of the new training program and curriculum.

Materials and Methods

Regional Hippology Contests

To assess youth knowledge related to equine science and the equine industry within Tennessee, scores were collected from each of the Tennessee Regional 4-H Horse Hippology contests. The Tennessee Regional 4-H Horse Hippology contest is hosted annually in each of the 3 regions in Tennessee (Eastern, Central, and Western) and consists of 3 phases (University of Tennessee Extension, 2023). The first phase is a written exam made up of multiple choice, true/false, and matching type questions. Participants in the junior age division are administered at 35-question written exam whereas competitors in the junior-high and senior age divisions receive a 50-

question exam. The second phase is a slides phase which requires participants to answer questions based on visual ques shown on a screen. During the slides phase, junior level participants answer 25 questions and those in the junior-high and senior divisions answer 50 questions. All questions for the written exam and slides phases are created by Tennessee Extension staff using approved resources. In 2021, the Tennessee Regional Hippology contest was administered in a full online format whereas the 2023 regional contest and the 2022 Eastern National contest were delivered in-person. Lastly, all participants compete in a judging phase where they must observe and place 1 halter class and 1 performance class. Specific content and topic areas represented in this contest is selected using the Tennessee 4-H Project Area Outcomes developed in 2018. Prior to data analysis, exam phase questions and slides phases questions were categorized into topic areas from the aforementioned project area outcomes. Regional contest data from 2021 were analyzed using frequency and means procedures in SAS 9.4 (Cary, NC) to determine a baseline and areas of knowledge deficiency. Analysis determined frequency of incorrect answers for individual questions along with average percentage of incorrect answers for each topic category. Additionally, areas of knowledge deficiency were identified based on this analysis and topic areas were omitted if not similar across all age divisions (Reproduction, Genetics, and Exercise Physiology). The topic areas represented included colors, behavior, reproduction, ownership, selection (S), breeds (B), facilities, hoof care, tack (T), health (H), nutrition (Nu), waste management, exercise, and genetics.

Youth (n=224) competed in the 2021 regional 4-H Hippology contest across the Eastern (n=59), Central (n=93), and Western (n=72) regions. Youth competed within their age divisions, where junior (grades 4 and 5, k=60), junior high (grades 6 to 8, k=100), and senior (grades 9 to 12, k=100) divisions competed in written exam and slide identification phases. Scores for each individual contestant were collected and placed into a spreadsheet according to their age division. Columns were labeled with question numbers and the topic area category in which the question was placed. For example, if question #1 pertained to hoof care, it was labeled “Hoof1.” Each row represented a different contestant, and they were given a “1” under each question they answered correctly and a “0” under each question they answered incorrectly. The same process was used to collect scores from the slides phase. Written exam questions and slide questions

were considered of equal value and were labeled with the same categories to determine overall performance in each knowledge area.

In January of 2023, data was collected from the 2023 Tennessee Regional Hippology contests where total participants (n=368) was determined along with overall participant numbers from the Eastern (n=113), Central (n=153), and Western (n=102) regions. Similar to the 2021 regional contests, youth competed within their age divisions, where junior (grades 4 and 5, k=131), junior high (grades 6 to 8, k=155), and senior (grades 9 to 12, k=82) participant numbers were collected. The scores from the 2023 contest were then compared with those from the 2021 contest to determine differences across age division, topic area, and county agent training status.

National Contest

The Eastern National Hippology contest is administered in a similar fashion to the regional level contests. However, this contest is only open to competitors in the senior age division who have won at their state level contest and is intended to have increased difficulty. Equine and youth extension specialists from participating states work in conjunction to develop contest material. In the 2022 Eastern National contest, 15 different states were represented. The national level contest still offers the same 3 written phases as the Tennessee Regional contests and administers a written exam phase and slides phase, but the question total is increased to 150 questions on the written exam in multiple choice format only. The question count for the slides phase remains at 50 questions similar to the regional contests. In addition, participants at the national contest much compete in a stations phase where their knowledge is tested in a more applied technique. Participants rotate through stations and answers questions before the allotted time expires. The stations phase consists of 15 stations with 10 questions at each station resulting in 150 questions answered in this phase. Similar to the Tennessee regional contests, all questions for the Eastern National Hippology contest are generated using the approved resources.

Written exam, slides, and stations phases from the Eastern National contest were categorized according to the Tennessee 4-H Project Area Outcomes similar to the categorization process

implemented for regional contest scores. Additionally, participant answers to each question were give a “1” or “0” for correct or incorrect answers respectively. Total participants in this contest (n=53) along with contest scores were collected in effort to compare areas of deficiency within Tennessee youth to those across the country.

Training Content Selection and Format

Once scores were collected and questions labeled with topic area categories, average percentage of correct and incorrect answers for each individual question were calculated in SAS v9.4 using the frequency procedure Upon making this calculation for all questions in the written exam and slides phases for the regional contest and the written exam, slides, and stations phases for the national contest, averages were calculated for questions of the same category. Once this step was completed, the topic areas with the highest percentage of incorrect answers were determined to be areas of knowledge deficiency and became the focus areas for this project. When choosing areas of knowledge deficiency, categories were omitted if they were not present across all age divisions or if they made up less than 5% of the overall composition of the questions (i.e. Genetics was omitted because it is only represented in the senior division and waste was omitted because there were minimal questions asked pertaining to this topic). Overall categories with lacking knowledge included Selection (S), Breeds (B), Tack (T), Health (H), and Nutrition (Nu).

Upon determination of areas of knowledge deficiency, a training program and associated curriculum was created for county Extension Agents to use when coaching their hippology teams for the 2023 contest. Subject matter experts were selected for each topic and were asked to create educational material associated with that topic to be given to all Extension Agents across Tennessee. Additionally, those subject matter experts developed an in-person training for agents that was implemented at the 2022 Joint Animal Science In-Service training. Agents in attendance not only received the same curriculum pieces but were also taught methods and strategies for implementing the curriculum to hippology teams in their home counties.

Participant Selection and Determination of Training Status

Annually, the University of Tennessee Department of Animal Science hosts an in-service training for county ANR and 4-H agents where they receive content and training regarding animal science topics across several species including horse, beef, dairy, small ruminant, and poultry. Agents from all 95 Tennessee counties are able to but not required to attend. In 2022, 51 ANR and 4-H agents of varying backgrounds and specialty areas were in attendance. Agents attending this training in 2022 were subjected to the train-the-trainer program we developed for this project and received the associated curriculum whereas agents not in attendance only received the curriculum component. The training module administered to agents in attendance delivered content on the specific knowledge gap subjects that were determined from the contest score analysis conducted on the 2021 regional horse hippology contest.

Final Data Set Analysis

The effects of age division, contest level (state or national), question category, and their interactions on student performance of answering test questions correctly were analyzed using mixed model logistic regression analysis with test results as the binary outcome variable. Student identifier was modeled as a random block effect. Multiple comparisons were performed with Tukey's adjust. Statistical significance was identified at $p < 0.05$. Analyses were conducted in SAS 9.4 TS1M8 for Windows 64x (SAS institute Inc., Cary, NC).

Results

Summary of Info/Introduction

Preliminary data were collected from the 2021 Tennessee 4-H Regional Hippology contests from each of the 3 regions of the state (Eastern, Central, and Western). For the junior age division, participants were exposed to 10 questions in the health category, 9 questions in the tack category, 11 questions in the nutrition category, 7 questions in the breeds category, and 10 questions in the selection category. For the junior high division, contestants answered 21 questions in the health

category, 13 questions in the tack category, 17 questions in the nutrition category, 7 questions in the breeds category, and 18 questions in the selection category. Finally, in the senior division, participants saw 24 questions in the health category, 9 questions in the tack category, 15 questions in the nutrition category, 3 questions in the breeds category, and 16 questions in the selection category. In 2023, the same questions were asked of the age divisions to maintain consistency. Therefore, the quantity of questions answered were the same across each age division (Table 6).

Effects Age Division, Category, Year, and Training Status

Significant effects were found when comparing across question category ($p < 0.0001$) (Table 7), age group ($p < 0.0001$) (Table 8), and year ($p < 0.0001$) (Table 9). Across all age divisions, the chance of students answering breeds questions correctly is 1.162 times as likely as students answering health questions correctly ($p = 0.0002$), 1.202 as likely as answering nutrition questions correctly ($p < 0.0001$), 1.042 times as likely as answering selection questions correctly ($p = 0.3258$), and 1.213 times as likely as answering tack questions correctly ($p < 0.0001$). Additionally, the chance of students answering health questions correctly is 1.035 times as likely as answering nutrition questions correctly ($p = 0.2539$), 1.044 times as likely as answering tack questions correctly ($p = 0.1935$), and 0.896 times as likely as answering selection questions correctly ($p = 0.0002$). Nutrition questions were 0.866 times as likely to be answered correctly when compared to selection questions ($p < 0.0001$) and 1.009 times as likely to be answered correctly when compared to tack questions ($p = 0.8005$). Additionally, selection questions were 1.164 times as likely to be answered correctly when compared to tack questions ($p < 0.0001$).

When comparing results across age divisions, junior level participants were 0.575 times as likely to answer questions correctly of any category when compared to junior high level participants ($p < 0.0001$). When comparing junior level participants to senior level participants, those in the junior

Table 6. Number of correct (1) and incorrect (0) responses across all years, categories, and age divisions

	Junior				Junior High				Senior			
	<i>2021</i>		<i>2023</i>		<i>2021</i>		<i>2023</i>		<i>2021</i>		<i>2023</i>	
	0	1	0	1	0	1	0	1	0	1	0	1
Breed	188	242	461	456	253	356	463	611	87	129	112	134
Nutrition	295	384	726	715	654	825	1164	1451	566	524	746	474
Health	246	371	740	570	740	1087	1505	1728	831	911	1032	922
Selection	285	331	733	577	619	947	1310	1460	427	732	563	742
Tack	300	253	720	459	478	653	906	1092	309	340	343	394

Table 7. Comparison across question categories of likelihood to have a correct answer

Category 1	Category 2	P Value	Odds Ratio
Breed	Health	0.0002	1.162
Breed	Nutrition	<.0001	1.202
Breed	Selection	0.3258	1.042
Breed	Tack	<.0001	1.213
Health	Nutrition	0.2539	1.035
Health	Selection	0.0002	0.896
Health	Tack	0.1935	1.044
Nutrition	Selection	<.0001	0.866
Nutrition	Tack	0.8005	1.009
Selection	Tack	<.0001	1.164

Table 8. Comparison across age division of likelihood to have a correct answer

Age Group 1	Age Group 2	P Value	Odds Ratio
Junior	Junior High	<0.0001	0.575
Junior	Senior	0.1892	0.924
Junior High	Senior	0.0003	1.221

Table 9. Comparison across year of likelihood to have a correct answer

Year 1	Year 2	P Value	Odds Ratio
2021	2023	<0.0001	1.308

age division were 0.924 times as likely to answer questions correctly ($p = 0.1982$). Additionally, junior high-level participants were 1.221 times as likely to answer questions correctly when compared to those in the senior age division ($p = 0.0002$) (Table 8).

An effect of year was also observed ($p < 0.0001$) when comparing across all age divisions. This effect indicated that participants were 1.308 times as likely to answer questions correctly in 2021 compared to 2023 (Table 9). Surprisingly, no effect was found when comparing across training status.

Comparison of Tennessee Regional Scores and Eastern National Scores

In addition to comparing performance across age division, year, and questions category, we also compared the performance of Tennessee senior level participants to those competing at the Eastern National Hippology contest. Overall performance was higher in senior level contestants in the Tennessee contests with 51.39% of answers being correct when compared to 40.47% of answers being correct amongst national contest participants. When comparing performance within each questions category between state and national level contestants, it was observed that state level participants were more likely to answer breeds, health, and selection questions correctly compared to those at the national level. However, there was no significant differences seen in performance across the nutrition and tack categories (Table 10).

Discussion

Study Origin

Across the state of Tennessee, Extension agents are located in each of the 95 counties to aid in carrying out the Land Grant mission of providing practical knowledge and skills to the common person (Duemer, 2007). However, the knowledge, background, and skills of these agents differ vastly leaving gaps in the knowledge base between agents and youth within each county. Specifically, many counties across the state lack agents with equine-specific knowledge and skills. Therefore, this study utilized the Tennessee Regional Hippology contests as a needs

Table 10. Comparison of percentage of correct responses from National and State contests across question categories

	State (%)	National (%)	p-value
Breeds	56.93	44.58	0.0242
Nutrition	43.20	36.66	0.0583
Health	49.59	37.80	<.0001
Selection	59.82	42.45	<.0001
Tack	52.96	47.45	0.3762

assessment for the Tennessee 4- H Horse program to determine which equine-specific topics reflected the lowest knowledge base amongst youth. Areas of deficiency were identified within youth contest participants and county agents were given the option to participate in the annual Joint Animal Science In-Service training to learn subject material and teaching strategies to take back to their county and deliver to their hippology teams and horse project groups. The purpose of this training was to implement the effective strategy of providing professional mentorship to educators by providing supplementation related to the identified areas of deficiency and, in turn, improve contest scores in those areas in the year following the training (Sood et al., 2016). However, this training is only offered once per year which could limit the attendance of agents and thus limit the overall effectiveness of the training when comparing scores from year to year. Additionally, the youth participants are not the same each year, meaning that many of the contestants from the 2021 contest would not have received the additional content and training strategies given to county agents prior to the 2023 contest. However, the overall number and demographic of participants were relatively similar with those from previous years.

Differences Between Age Divisions

Overall, junior-high level participants (6-8 graders) outperformed those of the junior level (4-5 graders). Likely, those participants in the junior age division have had little to no previous exposure to contest material or subject matter whereas those in the junior-high age division have some previous experience with the contest and the material contained within it. Therefore, it could be expected for junior level contestants to be outperformed by those of an older age group. Surprisingly, junior-high participants also outperformed senior level participants (9-12 graders). There are several factors that could influence this difference in performance. Firstly, question difficulty drastically increases between the junior-high and senior level where more in-depth and applied knowledge is required to do well in the senior level contest. This coincides with the learning strategies explained in Bloom's Taxonomy (Forehand, 2010). For example, senior level participants are required to know genetic coding for coat colors rather than just identifying a coat color based on phenotype. This could indicate the need to lower the overall difficulty of senior level questions or provide supplemental instruction at the junior-high level to better prepare

those participants for the new topics that are introduced when they are promoted to the senior age division. In order to combat the lack of performance from senior level participants, agents and educators may need to implement more effective teaching strategies to ensure that students are fully able to utilize the content. Higher order thinking strategies from Bloom's Taxonomy like "creating" and "evaluating" should be developed amongst senior level youth (Forehand, 2010). Additionally, this increase in presence of advanced concepts could result in lack of adequate training from some agents. For example, agents may not be familiar with genetic diseases, reproductive physiology, or exercise science and, thus, fail to teach those concepts to their senior level hippology teams. Additionally, it has been indicated that lack of time, lack of funding, and high work load present barriers for extension agents (Lakai et al., 2012). In some cases, county hippology teams are coached by outside volunteers rather than county agents. The same concept holds true related to the knowledge and skills of those volunteer leaders.

Differences Between Question Categories

Across all age divisions, results indicated a higher likelihood of participants answering breed questions correctly compared to all other questions categories. Likely, this is due to the material being relatively easy to understand and memorize. For example, there are a limited number of breeds, and they can be seen and memorized relatively quickly when compared to the more scientific concepts in other question categories. Alternatively, tack related questions reflected the lowest likelihood of being answered correctly by all age divisions. One explanation for this is the vast array of tack and equipment items that can be seen in the equine industry when comparing across the large variety of specific disciplines. Additionally, some of the more uncommon tack items such as pack saddles or driving equipment may not be the focus of agent instruction due to the lack of commonality in our geographic area. Another potential explanation for the lack of performance in the tack category is unintentional agent bias due to discipline familiarity. Horse savvy agents may compete or be more familiar with a certain discipline and thus focus more of their instruction toward tack and equipment related to that specific discipline. This could lead to participants lacking equal knowledge of tack and equipment across multiple disciplines. Despite the lack of research pertaining to performance within youth livestock and equine educational

contests, these explanations align with previous findings that content contained within the question itself is just as influential as question or stimulus format when evaluating learner performance via written or visual exam (Schuwirth & Van Der Vleuten, 2004).

Differences Between Year and Training Status

Surprisingly, performance in areas of deficiency was greater in 2021 when compared to 2023 and no effect of training status was observed. Previous research has indicated that train-the-trainer program prove successful across a variety of industries, including agriculture (Bess et al., 2003; Carruth et al., 2010; Orfaly et al., 2005; Pancucci, 2007; Sood et al., 2016). Therefore, it was expected that scores would improve between years due to the implementation of agent training and curriculum development. There are several potential explanations for this result. Firstly, contest structure could play a vital role in the overall performance of the youth. In 2021, the Tennessee Regional Hippology contests were held virtually due to the COVID-19 pandemic. During this period, contestants completed the written exam and slide identification phases of the contest through a Google Form. On the other hand, the 2023 contests were held in-person as they traditionally have been where contestants answer questions from a physical copy of the written exam and view slides in-person. Research has shown that learners engaging in strictly online education platforms have equal or increased success and satisfaction when compared to face-to-face models (Huang, 2020; Neuhauser, 2002). Therefore, participating in a strictly virtual contest could have provided students with comfort or lack of nervousness that is typical with in-person competition and, thus, resulting in higher overall scores.

Additionally, there is the potential of a “lag effect” on the training provided to the agents. The agent training and introduction of supplemental curriculum was provided in December of 2022 prior to the regional contests that took place in February of 2023. It is likely that coaches provided instruction on these target content areas prior to receiving the training, causing them to implement the new material in the following year. Finally, agent turnover could also play a role in affecting the overall scores in the observed areas of deficiency. For example, an agent could have received the training and curriculum but left their county position prior to implementing it.

Therefore, participants in those particular counties would not have received instruction from the new training and curriculum presented to the agents at the in-service training.

Difference in Senior Scores Between State and National

An overall difference in performance was observed when comparing senior level competitors from the Tennessee state contests to those in the Eastern National contest. It was shown that state level participants had higher performance in all question categories. Most likely, this is due to the significant increase in content difficulty between the Tennessee regional contests and the Eastern National contests. Additionally, the structure of the contests is slightly different. At the Eastern National contest, participants must rotate through stations and answer questions independently without the ability to converse and seek help from their teammates, thus adding increased stress, nervousness, autonomy.

Limitations

The Tennessee regional hippology contests were delivered in different formats in 2021 and 2023. The COVID-19 pandemic resulted in the 2021 contests to be delivered in a fully online platform as opposed to the in-person format of the 2023 contests. This could skew performance results due to participant preferences and learning styles related to online and in-person delivery. Despite this limitation, data collection from this study has the potential to provide comparative information on hippology contestant performance in an online setting vs. an in-person model. The literature suggests that performance is not negatively affected when engaging in a strictly online learning model as opposed to a fully face-to-face approach (Neuhauser, 2002). Conversely, learners commonly prefer a face-to-face setting, so future research is warranted to determine if the contest structure impacted contestant performance (Allen et al., 2002; Carrol & Burke, 2010).

Additionally, the set of youth that participated in the 2021 contests and the 2023 contests are likely composed of different individuals due to participants aging out, new youth entering the

contest, and teams being comprised of different youth sets. Thus, we were not able to compare knowledge retention on an individual basis. However, collecting data from different sets of youth provides a more all-encompassing representation of youth performance within the hippology contests along with data that is more reflective of agent training effect.

Agent turnover is another factor that could be a potential limitation to this study. Within the state of Tennessee, it is common for counties to see changes in agent employees frequently.

Furthermore, this high turnover rate could have left some counties without equine-focused programs and create new horse project in other counties between 2021 and 2023, creating inconsistencies within the knowledge base of Tennessee youth. Additionally, newer agents may be unfamiliar with the process of accessing horse project and hippology resources through SharePoint and could cause their hippology teams to lack adequate training and knowledge. Despite the high agent turnover rate and common unfamiliarity with hippology resources, this project succeeded in providing participating counties with valuable knowledge, skills, and resources to bring back to their counties to implement within their hippology teams and 4-H horse project groups.

The initial timeframe of training program implementation could prove to be another limitation of this study. The lack of an observed training effect could be accredited to the training being administered too close to the regional contest, prohibiting agents from using the materials and strategies learned at the training before the students competed. However, the training is still provided agents with valuable curriculum and strategies to implement in the future. Future evaluation of contest scores could yield a training effect due to the additional time granted for agents to implement the aforementioned materials and teaching strategies. Additionally, future data collection over a span of multiple years could prove helpful in making more meaningful conclusions based on training impact and repeatability.

Implications and Conclusions

Findings hold immense value when assessing the current state of the Tennessee 4-H Horse Project and could prove to be invaluable across other youth contests if applied. The methods used in this study can provide valuable insight into knowledge gaps within youth regardless of the subject matter or content area. Additionally, if implemented yearly, this model could prove useful in providing long term tracking of performance data for any youth organization, contest, or project group both within and outside of an extension setting. Our findings yielded that participant scores did not significantly increase after the implementation of the train-the-trainer program. However, this data still holds immense value in evaluating the knowledge status of Tennessee 4-H Hippology contestants and can provide a framework for future train-the-trainer programs and knowledge assessments for youth livestock and equine programs.

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VITA

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