IDENTIFYING BREED DIFFERENCES IN INSULIN DYNAMICS, SKELETAL MUSCLE
AND ADIPOSE TISSUE HISTOLOGY AND GENE EXPRESSION

By

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ABSTRACT

IDENTIFYING BREED DIFFERENCES IN INSULIN DYNAMICS, SKELETAL MUSCLE AND ADIPOSE TISSUE HISTOLOGY AND BIOLOGY

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Equine metabolic syndrome (EMS) and associated insulin dysregulation (ID) have been identified as the most common causes of laminitis. Certain breeds seem susceptible to EMS, and we have identified breed differences in metabolic phenotypes. Muscle and adipose tissue have important roles in glucose and insulin regulation, but little is known about how their biology affects breed-related insulin sensitivity and other metabolic traits.

In chapter 2, breed specific differences in insulin and glucose dynamics during three dynamic challenge tests for diagnosing EMS/ID were evaluated. An arginine stimulation test (AST), an oral sugar test (OST) and a frequently sampled insulin modified intravenous tolerance test (FSIGTT) were performed in 27, 82, and 90 individuals representing five different breeds (Quarter Horses [QH], Arabians, Morgans, Welsh Ponies [WP], and Thoroughbreds [TB]). The AST (70 mg/kg bwt intravenous dose of arginine hydrochloride) elicited a significant increase in insulin concentrations in adult horses, which lasted at least 15 minutes and was repeatable. During the OST, insulin but not glucose was useful for diagnosing ID, and insulin thresholds to diagnose ID, which are lower than previous recommendations were established. Longitudinal analysis of insulin and glucose trajectories demonstrated that breed, age, triglycerides, and high molecular weight adiponectin were all associated with differences in the shape of the insulin and/or glucose curve. Minimal model analysis of the FSIGTT was performed and compared between breeds, with QHs having some of the highest insulin sensitivities (SI).
In Chapter 3, tail head adipose tissue (TAT) and gluteal muscle biopsies were performed in a cohort of horses and ponies for to identify differences in histologic traits and to evaluate these traits with respect to SI. Overall, measures of adiposity, adipocyte size, and muscle fiber type did not have strong correlations to tissue level SI and the acute insulin response to glucose providing further evidence that horses can demonstrate both a metabolically healthy obese, as well as metabolically unhealthy thin phenotypes. Breed differences existed in adipocyte area, with QH having a significantly smaller mean adipocyte area than both Arabians and WP but not TBs or Morgans. Muscle fiber type total percent area and proportion did not correlate to SI. QH did have a greater area of type 2B to 2A muscle fibers than type 1 fibers. Fiber type area and proportions of fiber types did not significantly differ between breeds.

In Chapter 4, middle gluteal muscle and TAT biopsies obtained from 28 geldings from four breeds were examined for differential gene expression and functional analysis using RNASeq and Ingenuity Pathway Analysis. Each breed uniquely differentially expressed genes in each tissue (7-1347 in adipose, 94-691 in muscle). In TAT, top networks in Arabians and WP were Carbohydrate Metabolism and Developmental Disorders/Lipid Metabolism respectively. Arabians had upregulation, and WP down regulation of peroxisome proliferator activated receptor, co-activation receptor 1. Novel genes and pathways were determined and breed specific patterns of differentially expressed genes may contribute to ID.

Unifying themes of Chapters 2-4 were the effect of breed on EMS defining traits, and the need to evaluate the larger picture (systemic, histologic, and transcriptomic) to better understand the true phenotype of a horse or pony that is susceptible to EMS/ID.
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1. Introduction and Literature Review

Introduction

Although an American College of Veterinary Internal Medicine (ACVIM) consensus statement defined EMS by 1) generalized obesity and/or regional adiposity, 2) insulin resistance (IR)/insulin dysregulation (ID),[1] 3) dyslipidemia,[1] and 4) laminitis, descriptions of the metabolic phenotype of laminitis-prone horses and ponies vary between studies and recent evidence suggests that much of this variability is due to breed differences.[2-6]. Further, while several studies have described the whole horse phenotype and insulin and glucose dynamics, little is known about the underlying histologic and molecular pathophysiology of the syndrome. This thesis advances the knowledge about this syndrome beyond diagnosis based on clinical blood testing and morphometric descriptions by examining the cellular and molecular phenotypes underlying the metabolic dysfunction(s) in EMS. The work outlined here aims to gain novel insights into the breed specific tissue and molecular level pathophysiology which has been little interrogated to date, in order to better inform choice and interpretation of blood testing and to further define the EMS phenotype(s). In order to do so, five breeds of horses will be characterized across 3 phenotypic levels: 1) clinical measures of whole body insulin and glucose dynamics, adipokines, and dyslipidemia; 2) skeletal muscle and adipose tissue histopathology; and 3) skeletal muscle and adipose tissue gene expression. The 5 breeds were selected for differing susceptibility to EMS: 3 high-risk breeds, the Arabian, Morgan, and Welsh Pony (WP); and 2 low-risk breeds the Quarter Horse (QH) and Thoroughbred (TB). The overarching aims of this thesis are to detect breed differences in EMS metabolic phenotypes across the whole body, tissue and transcriptomic levels and to recognize the relationships between these phenotypic
levels in order to identify which combination of whole body and phenotypic markers are best to achieve a diagnosis, allowing earlier management changes to prevent laminitis.

**Review of relevant literature**

**Current definition of Equine Metabolic Syndrome**

Equine metabolic syndrome (EMS) has been defined by the following cluster of clinical signs:

1) generalized obesity and/or regional adiposity,

2) insulin resistance (IR)/insulin dysregulation (ID) characterized by hyperinsulinemia and/or abnormal insulinemic responses to oral or intravenous (IV) glucose,[1] and

3) laminitis that develops in the absence of other recognized causes.[6-9]

Recent studies have demonstrated that hypertriglyceridemia and low serum high molecular weight (HMW) adiponectin are other consistent features of the EMS phenotype. [1,9-12] As shown below, EMS horses are identified via a combination of these phenotypic, morphometric, and static and/or dynamic tests.

*Generalized Obesity and/or Regional Adiposity*

Generalized obesity and/or regional adiposity has been the mainstay of the phenotypic description of a horse suffering from EMS.[6] Areas of regional adiposity commonly encountered are: neck, ribs, behind the shoulder, back, preputial/mammary gland, and tailhead region.[6] The Henneke[13] body condition score (BCS) has been used since the 1980’s to subjectively describe a horse’s body fat distribution. For assessment of regional adiposity, a cresty neck score has been used in several studies and appears to demonstrate a breed
predilection.[14-16] More recently, the work of Dugdale et al. (2012) has provided a more accurate description of the true relationship between BCS and total body fat mass (TBFM) by comparing BCS to morphometric measurements of heart girth to withers height ratio and adipose dissection from cadavers.[17] This investigation determined that BCS has an exponential relationship to TBFM, making it an insensitive predictor of TBFM for horses that are overweight or obese.[17] Because of the need for a more accurate estimation of TBFM in the horse, a deuterium dilution test used to estimate more accurately total body water and fat in horses was found to strongly correlate to measurements derived from cadaver dissection. [18] More recently, bioimpedence spectroscopy has been evaluated for determination of body composition in a group of Standardbred horses. However, due to the underestimation of total body water, and overestimation of TBFM by this technique, the applicability of bioimpedence spectroscopy to the horse may be limited.[19] While obesity and/or regional adiposity has been considered a hallmark of EMS, increasingly there is evidence in human studies of the existence of “fat but fit” or metabolically healthy obese (MHO) individuals as well as lean and metabolically unhealthy individuals.[20] It is unknown whether or how frequently this phenomenon may occur in horses and further evaluation of possible relationships between adiposity and insulin sensitivity are needed.

*Insulin Resistance (IR)*

Insulin resistance describes a condition where there is a decreased response of the tissues (eg. muscle, liver, or adipose) to normal physiologic amounts of circulating insulin. As such, the beta cells of the pancreas secrete greater quantities of insulin to produce a biologic function. One example would be the requirement for greater amounts of insulin to stimulate glucose
transport into cells. In this instance, glucose uptake is reduced as a result of impaired GLUT4 translocation to the cell surface in skeletal muscle.[21]

Historically, IR has been associated with metabolic syndrome in humans and horses.[6,22,23] The presence of IR is an important pathophysiologic component of EMS,[6,22,24,25] as evidenced by the reported clinical association between the hyperinsulinemia in insulin resistant animals and incident laminitis, as well as the experimental induction of laminitis following 48 hours of euglycemic-hyperinsulinemia in previously normal horses. [26,27] In both cases, an episode of acute exacerbation of hyperinsulinemia (either dietary or induced respectively) is thought to cause the laminitic event. The dietary trigger for the laminitic event in horses with chronic resting hyperinsulinemia may not have been sufficient to cause laminitis in an individual that had normal resting insulin levels however, although this is unproven. These clinical experiences and research studies emphasize the need for practical and reliable tests for evaluation of insulin dynamics in horses.

*Insulin Dysregulation (ID)*

This term has been newly introduced and encompasses abnormal fasting hyperinsulinemia, excessive insulin response to oral or intravenous sugar administration, as well as evidence of IR.[1,28-30] This definition reflects the idea that hyperinsulinemia can occur independently of IR and is not just a sequela of IR. As hyperinsulinemia has been linked to laminitis,[31,32] understanding of how to best assess insulin dynamics in horses, particularly responses to an oral challenge that would mimic what occurs in nature while gazing on pasture,[29] is vital to better diagnosis and early treatment. Examining the similarities and/or differences between insulin and glucose responses to oral versus intravenous (IV) challenges are
also important steps towards understanding insulin dynamics in horses, further elucidating possible underlying pathologies.

Dyslipidemia and Altered Circulating Adipokine Concentrations

Triglycerides (TG) are stored in adipose tissue in response to insulin. In the case of an insulin resistant individual, insulin signaling is ineffective, so protein kinase B (Akt) is not activated, which results in activation (and not inhibition) of protein kinase A (PKA). This leads to dyslipidemia and further impairment of insulin signaling.[33] The increased non-esterified fatty acids (NEFA) levels result in increased TG synthesis and secretion from the liver. Hypertriglyceridemia has been associated with hyperinsulinemia in ponies,[34] and elevated levels of NEFAs[10] have been found to be higher in obese, insulin resistant horses versus non-obese animals.[10]

Adipose tissue is now recognized to be a highly active endocrine organ, and leptin and adiponectin are two key adipokines that are primarily expressed by adipocytes.[35] Leptin, a hormone regarded as the “satiety hormone”, has been associated with IR in the horse,[10] and with hyperinsulinemia in ponies,[34] but appears, for the most part, to correlate positively with increasing body weight and TBFM.[3,36] Comparisons between obese horses with IR and non-obese horses determined that resting insulin, leptin, NEFAs, very low-density lipoproteins and high density lipoprotein-cholesterol concentrations (HDL-C) were higher in the former group.[10] Resting insulin, leptin, and TGs were also reported to be elevated in IR obese horses in another study.[37] However, differences in leptin concentrations have been reported between horses and ponies, and between breeds, [14,38] which may limit the ability to generalize these data. Finally, gender differences in circulating leptin concentrations have been noted, although
reports are conflicting with some authors reporting higher concentrations in mares,[39,40] while others report higher concentrations in geldings,[41] or no detectable differences between genders.[38] Thus leptin’s utility as a biomarker for metabolic dysfunction and EMS is questionable.

In contrast to leptin, adiponectin concentrations are inversely proportional to body mass, and adiponectin is considered insulin sensitizing, making adiponectin of interest as a possible screening test for EMS. Adiponectin is released from adipose tissue in both low molecular weight (LMW) trimers and high molecular weight (HMW) multimers. There is also a globular proteolytic fraction of the protein.[42] The HMW form of adiponectin is considered the most metabolically active with the overall decrease in adiponectin concentration seen in obese human patients due to a decrease of the HMW form.[43] Low levels of adiponectin have been associated with ID in the horse,[3] as well as being a predictor for future laminitic events.[44] Measures of total adiponectin have been significantly associated with laminitis development in ponies.[44] The globular form of adiponectin also promotes glucose uptake and lipid oxidation while enhancing insulin signaling and myogenesis in humans but has an unknown role in the equine.[42] The ELISA for HMW adiponectin assay that was used in this study,[45] has been removed from the commercial market, however, a radioimmunoassay that measures total adiponectin is still available.[44,46]

**Laminitis**

Laminitis is the primary outcome of concern with EMS and one of the primary reasons for a veterinarian to be called out to a farm to examine a horse,[47-49] with up to 15% of farms with horses nationwide reporting at least one laminitic episode annually.[47] Although laminitis
has several inciting causes, surveys of equine veterinarians have indicated that endocrinopathic disorders (EMS and Pituitary Pars Intermedia Dysfunction [PPID]) are the most common cause of laminitis within their practices.[47] Hyperinsulinemia in EMS individuals is likely the clinical phenotypes most important for laminitis development. Hyperinsulinemia has been implicated as a causal factor in endocrinopathic laminitis development ever since it was demonstrated that prolonged induced hyperinsulinemia resulted in onset of laminitis in ponies and Standardbreds.[31,32,50,51] This finding has reinforced the need to develop testing protocols that can best assess insulin and glucose dynamics in order to better diagnose, and thus help prevent, this painful disease which can result in loss of use or euthanasia.[52,53]

**Diagnostic testing for the components of Equine Metabolic Syndrome**

*Static Testing*

Static testing for EMS typically involves blood collection the morning after only leaving one flake of hay in the stall at 10 PM. These single sample collections are considered screening tests, with a fasting glucose concentration of >110 mg/dL, a fasting insulin of >20 μU/mL, and/or a fasting leptin of >7ng/mL being considered abnormal.[6,14] Recent studies have also evaluated the roles of NEFAs, TGs, adiponectin as possible static screening markers. [10,45] The sensitivity of these static tests, using insulin sensitivity status as the gold standard, have been called into question:[54] however, because static tests are simple to perform (i.e. a single blood sample), there is continued interest in their use. This interest has lead to the development of several calculated indices used in humans which can improve sensitivity for detecting IR or ID (indices such as the reciprocal inverse square of basal insulin [RISQI], MATSUDA, insulin sensitivity in the oral glucose tolerance test [SIisOGTT], and Avignon for example). Poor
sensitivity of static tests has also lead to increased use of dynamic testing, particularly dynamic
tests that can be performed quickly on the farm with the use of a hand-held glucometer.

**Dynamic Testing**

Dynamic challenge tests can take three forms:

1. those that provide insight into tissue sensitivity to exogenous insulin;
2. those that examine the acute insulin response (AIR) of the pancreas; and
3. those that evaluate both tissue insulin sensitivity and pancreatic response

Table 1.1). Dynamic tests are believed to better approximate post-prandial insulin and

sensitivity to exogenous insulin that have been performed in

Tests that examine the tissue sensitivity to exogenous insulin that have been performed in

Tests that examine the AIR include: the first twenty minutes of an FISGTT (AIR to IV glucose [AIRg]), an intravenous
glucose tolerance test (IVGTT),[60] the arginine stimulation test (AST, the AIR to arginine
[AIRarg]), the oral sugar test (OST),[11,61,62] the oral glucose tolerance test (OGTT)[57], the oral glucose test (OGT)[63] and the in-feed oral glucose tolerance test (in-feed OGTT).[29,64]

Table 1.1 Descriptions and comparisons of different dynamic challenge tests for the diagnosis of EMS and IR/ID horses. Intravenous (IV), oral (PO), Insulin resistant (IR), Insulin dysregulated (ID), Insulin Sensitivity (SI), Acute Insulin Response (AIR), Area under the curve (AUC), Oral Sugar Test (OST), Euglycemic hyperinsulinemic Clamp (EHC), Combined Insulin and Glucose Tolerance Test (CGIT), Oral Glucose Test (OGT), two-step Insulin Tolerance Test (2SIIT), Arginine Stimulation Test (AST), intravenous Glucose Tolerance Test (IVGTT), Oral Glucose Tolerance Test (OGTT)

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<tr>
<td>EHC</td>
<td>SI</td>
<td>IV</td>
<td>SI_{clamp} based on insulin stimulated glucose uptake (M), change in insulin concentration and glucose concentration during steady state[57,65] The EHC has lower interday variation in SI than the FSIGTT.[65] M value lower in ponies.[66]</td>
<td>See FSIGTT</td>
</tr>
<tr>
<td>2SIIT</td>
<td>SI</td>
<td>IV</td>
<td>IR if glucose not decreased by ≥50% in 30 min[58]</td>
<td>Comparable to the complete insulin response test[58]</td>
</tr>
<tr>
<td>CGIT</td>
<td>SI</td>
<td>IV</td>
<td>IR if: Glucose does not return to baseline by 45 min and insulin is &gt;100uU/mL at 45 min[67] Insulin, but not glucose, curves were highly repeatable and breed affected glucose results (Icelandic horse vs Standardbred).[59] Glucose slope 1-45 min, not insulin useful for diagnosis.[68]</td>
<td>With the FSIGTT as a standard for classifying horses as IR, the CGIT had a Sensitivity:85.7% and Specificity 40% for glucose, insulin was worse for sensitivity (28.5%).[54] Less horses were classified as IR compared to an OGT.[29]</td>
</tr>
<tr>
<td>IVGTT</td>
<td>AIR</td>
<td>IV</td>
<td>Donkey responses differ from horses.[69] SI was calculated as for the FSIGTT.[60]</td>
<td>See the AST comparisons.</td>
</tr>
<tr>
<td>AST</td>
<td>AIR</td>
<td>IV</td>
<td>Adult horses have an insulin response to arginine[70] Pony foals were IR at one day old and had an insulin response to arginine.[71]</td>
<td>Ponies had a lower insulin response to the AST as compared to the IVGTT.[71]</td>
</tr>
<tr>
<td>Test</td>
<td>Type</td>
<td>Dosage</td>
<td>Description</td>
<td>Notes</td>
</tr>
<tr>
<td>------</td>
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<td>--------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>OST</td>
<td>AIR</td>
<td>PO</td>
<td>IR if insulin &gt; 60 mU/mL or glucose &gt;125 mg/dL at 60 or 90 min[11] Poorly repeatable, fasting exacerbated insulin responses, but fed responses were similarly diagnostic.[62] Fast horses for 3 hours before the OST [61]</td>
<td>AUCglucose correlated between OST, CGIT, and FSIGTT, but overall lack of agreement, OST highly specific.[54]</td>
</tr>
<tr>
<td>OGT</td>
<td>AIR</td>
<td>PO</td>
<td>1 g/kg via gavage[57] 1.5 g/kg, breed differences seen, peak and AUC insulin higher in ponies and Andalusians than Standardbreds.[72]</td>
<td>Strong correlation of insulin at 120 min to SIclamp[57]</td>
</tr>
<tr>
<td>OGT</td>
<td>AIR</td>
<td>PO</td>
<td>0.75 g/kg PO, &gt;90 μIU/ml 2 or 4 h after feeding= ID[29] Overall insulin responses were repeatable, with individual responses insulin varied more than glucose. The 90 min time point was consistent.[63]</td>
<td>Compared to the IVGTT, the OGT identified more ponies with ID[29]</td>
</tr>
<tr>
<td>In-feed OGT</td>
<td>AIR</td>
<td>PO</td>
<td>1 g/kg PO, time of max insulin differs between horses and ponies.[64]</td>
<td>Identified more horses as IR than the OST, overall 85% agreement, higher AUCinsulin[64]</td>
</tr>
</tbody>
</table>

**Breed differences in susceptibility to EMS, insulin sensitivity, and insulin and glucose dynamic testing**

Insulin resistance/insulin dysregulation is thought to be an important risk factor for development of laminitis, to which, anecdotally, certain breeds appear predisposed (Morgans, Arabians, Tennessee Walking Horses, Andalusians, Icelandic Horses, Dutch Warmblood and ponies in general) while others breeds do not (QH, Standardbreds, Thoroughbreds).[6,10,66,73-76] Understanding breed differences in various biological markers important to EMS in response to dietary trials and/or static/dynamic testing is important for early EMS diagnosis and appropriate intervention.
Breed related differences in insulin sensitivity, insulin responses to a meal containing glucose, and an oral glucose tolerance test (OGTT) have been investigated in Standardbreds, Andalusians, and ponies.[72] Ponies and Andalusians had higher peak insulin responses as well as an increased area under the curve (AUC) for insulin during the OGTT, and a lower insulin sensitivity (SI) when compared to Standardbreds.[72] In another study evaluating that examined these same breeds, insulin, glucose, and GLP-1 responses to a corn meal, higher insulin and GLP-1 AUC was noted in Andalusians and ponies when compared to Standardbreds. Glucose was not significantly different between breeds in either study.[72,77] A third study in which these same breeds were fed either cereal- or fat-rich meals, ponies and Andalusians were determined to have a lower SI than Standardbreds. In another study that compared a more IR breed (Icelandic horses) to a typically insulin sensitive (IS) one (Standardbreds), the investigators determined that during a CGIT, a breed difference in glucose but not insulin dynamics was detected.[59] Whereas both breeds experienced a period of hypoglycemia during the test, a more prolonged rate of decrease in plasma glucose was evident in Icelandic horses when compared to Standardbreds.[59]

Differences in insulin and glucose dynamics are also evident between breeds that have more traditionally been considered to be insulin sensitive. Our laboratory, in collaboration with the University of Minnesota, investigated differences in insulin and glucose concentrations during an FSIGTT and in minimal model analyses of the FSIGTT in Standardbreds, QHs, and TBs.[4] In this study, Standardbreds, unlike the QHs and TBs, were able to maintain appropriate glucose levels and avoid periods of hypoglycemia, suggesting a more rigorous regulation of glucose homeostasis.
Researchers at the University of Minnesota, in a large cohort of animals (N=634) of multiple breeds, found QHs to have lower baseline insulin and insulin post OST (75 minutes), as well as lower TG and leptin when compared with other breeds.[5] In this same group of animals, WP were found to have a greater insulin response to the OST than Morgans, and Morgans, Tennessee Walkers, and other high risk breeds had baseline insulin concentrations greater than QHs, with ponies higher than both QHs and Arabians. For the OST insulin response, all breeds were higher responders than QHs. Leptin concentrations were higher in Morgans, ponies, and high risk breeds than QHs, and Morgans had higher leptin concentrations than the low risk breeds. QHs had higher adiponectin concentrations than Arabians.[5]

Muscle and adipose histology

Muscle histology and relationship with insulin sensitivity and breed

There is conflicting information about the relationship between muscle fiber composition and insulin sensitivity. In one study in human subjects, type I (oxidative) muscle fibers were demonstrated to have a higher glucose handling capacity than type II fibers, but both fibers were found to be similarly sensitive to insulin.[78] Another study in humans by Stuart et al. (2013) found a decreased proportion of type I fibers with an increased proportion of type IIA (mixed oxidative and glycolytic) to IIB (glycolytic) muscle fibers was associated with metabolic syndrome.[79] However, it remains to be determined whether either of these associations holds true in horses.

While breed differences in percentages of muscle fibers in different muscles have been noted,[80] information examining those percentages in relation to insulin sensitivity differences between breeds is lacking. Available information indicates that QHs, typically considered an insulin sensitive (IS) breed, are reported to have a greater proportion of type IIB to type I fibers;
Belgian Horses have a greater proportion of type 2a versus 2b type fibers as compared to QHs. QHs with polysaccharide storage myopathy (PSSM) were also noted to have increased insulin sensitivity; however, the control group in this study were Thoroughbreds and not QH without PSSM, so breed is likely contributed to this difference as QHs are generally more IS than TBs. This suggests that, unlike the investigation of human subjects by Stuart et al. (2013) referred to previously, a greater proportion of IIB fibers are desirable, and is discordant with the idea in the human literature that a greater proportion of type I (oxidative) fibers drives improved insulin sensitivity. This fiber proportion pattern and increased insulin sensitivity has also been seen in myostatin (MSTN) knock out mice and cattle. In mice, it has been proposed that myostatin regulates fiber-type composition by controlling myocyte enhancer factor 2 (MEF2C) and MyoD expression, with MSTN knock outs having increased MyoD expression. This may also occur in horses as many QHs have a MSTN gene variant that results in a decreased expression of the myostatin protein as is further described below in section 1.2.5.

Adipose tissue histology and relationships with insulin sensitivity and breed

A current theory about IR and obesity states that with excess caloric intake, long chain fatty acids accumulate in other organs once the adipocyte is saturated. Additionally, the adipocyte hypertrophies until it outstrips its blood supply from the extracellular matrix. At this point, the adipocyte releases adipokines (Tumor necrosis factor alpa (TNF alpha); Monocyte Chemoattractant Protein-1 (MCP-1)) and other inflammatory attractants, which has led to the implication of adipocyte size, specifically hypertrophy, as a risk factor for type 2 diabetes in humans and to an association with higher fasting insulin levels.
In humans, certain adipose tissue depots (the subcutaneous and visceral abdominal depots) more commonly associated with metabolic syndrome contain hypertrophied adipocytes, which undergo lipoapoptosis releasing inflammatory mediators.\[95\] In the horse, there is limited data on adipocyte size and presence of macrophages or inflammatory mediators in different adipose tissue depots. Currently, the study by Bruynsteen et al. (2013) \[96\], provides the only published information regarding adipocyte size in different depots (mestenteric, nuchal, peri-renal, tail head, and retroperitoneal) in the horse, with peri-renal adipocytes having the largest cross-sectional area. The nuchal adipose depot was considered closest in biological behavior to the human subcutaneous abdominal depot. This study utilized a variety of breeds of horses of varying age, and BCS, thereby prohibiting examination of breed differences. As blood analysis was limited to resting glucose, insulin, and leptin concentrations, the relation of adipocyte size to insulin sensitivity could not be determined, although correlations between adipocyte area and mRNA expression in inflammation-related genes were noted and are discussed later in this chapter.\[96\] In one cohort of horses and ponies fed a glucose diet to induce obesity over the course of 20 weeks, increasing BCS was not associated with a decrease in SI, which would appear to indicate that the pathology of metabolic syndrome in horses may differ from humans.\[3\]

**Muscle and adipose tissue gene expression and the relationship to metabolic dysfunction**

*M.*Myokines and Adipokines

Myokines and adipokines are groups of cytokines that largely originate in muscle or adipose tissue respectively. The first myokine to be identified was myostatin,\[97\] which is a
member of the TGF-beta family and a negative regulator of muscle growth. IL-6 is a myokine that has proportionally increased expression with increasing exercise and muscle mass, but which can also be increased and enter the circulation with obesity.[98] Other myokines include the anti-inflammatory interleukin-10 (IL-10) and IL-1 receptor antagonist.[99]

A myokine of recent interest is irisin, which is cleaved from Fibronectin type II domain containing protein 5 (FNDC5), although some have called its existence in the horse and other species (bovine, mouse and human) into question.[100] Decreased levels of myostatin are thought to allow increased concentrations of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1alpha), and thereby FNDC5 (which is downstream from it). Removal of the transmembrane domain releases a fragment of the protein named irisin. Irisin affects adipose tissue by causing an increase in uncoupling protein (UCP)-1, resulting in white adipose tissue taking on brown adipose tissue characteristics (noted by increased Tbx1 and Tmem26 which are mRNA markers of beige adipocytes).[86,101] Myostatin inhibition also reduces inflammatory cytokines in muscle and adipose, while stimulating fatty acid oxidation.[101] Local myostatin inhibitors can also result in increased skeletal muscle glucose dispersal, ameliorating IR.[102] In metabolic syndrome patients on an energy restricted diet, reduction of irisin corresponded with decreases in lipid metabolism, minimizing one of the clinical signs of metabolic syndrome.[103]

The concentration of adipokines (commonly adiponectin and leptin) is frequently examined for correlations to IR and ID; however, IL-6, apelin, monocyte chemotactic protein-1 (MCP-1) and TNF-alpha also have roles in inflammation and possible promotion of IR.[104,105] IL-6 can have both pro- and anti-inflammatory effects depending on its tissue of origin (adipose is pro-).
The roles of myokines and adipokines in horses are in the early stages of understanding, with the latter being the most investigated to date with regards to EMS. A single nucleotide polymorphism intron 2 of MSTN[106,107] has been implicated as a powerful predictor of racing performance. This intronic SNP has known been shown to be a marker of the true functional variant a short interspersed element (SINE) in the MSTN promoter which disrupts MSTN gene expression.[90] Similar to the metabolic changes in MSTN knock-out mice, SINE insertions present in the myostatin gene promoter region of Quarter Horses has been linked with an increase in type IIb muscle fibers as mentioned above, as well as to higher adiponectin concentrations, and lower leptin, basal insulin, and insulin during an OST (NE Schultz and ME McCue unpublished data). Decreased myostatin expression has been linked to increased insulin sensitivity in bulls.[108]

In humans, different adipose tissues have different inflammatory characteristics, with visceral adipose depots containing greater inflammatory cytokines than subcutaneous (SQ) depots (with the exception in some instances of the abdominal SQ depot).[109] Investigations into adipose tissue depot differences (SQ vs visceral sites) in adipokines in IR and IS horses, noted that the nuchal ligament (SQ depot) had elevated levels of mRNA expression of IL-1beta and IL-6, but not TNF-alpha and MCP-1.[110] This finding would differ from humans and other species where the visceral adipose depots demonstrate the most inflammatory mediators. Another study in horses did not detect significant differences in IL-6 by depot but determined that there were inconsistent differences in inflammation-related genes between depots.[96] However, this finding was based on 12 horses of different breeds, ages, and body condition types all of which are factors that may have confounded the analysis.
EMS related muscle and adipose gene expression studies

Gene and protein expression studies of equine skeletal muscle and adipose tissue for the most part have been based on a single breed or small numbers of many breeds and limited to just a few genes or proteins of interest. (Table 1.2)[7,60,96,110,111] In horses, recent work has examined gene expression in EMS horses in muscle and adipose tissue. Researchers examining gene expression in equine skeletal muscle in EMS horses did not find any association between markers of inflammation or oxidative stress and obesity.[112,113] Researchers examining gene expression in the nuchal adipose tissue in EMS horses/ponies found increased levels of interleukin (IL)-6 but not of tumor necrosis factor alpha (TNF-alpha) when compared to non-EMS obese controls.[114] Other studies did not demonstrate differences between EMS and non-EMS horses and ponies in regards to gene expression, but did see depot differences in gene expression, with the nuchal adipose depot containing higher amounts of IL-1beta and IL-6.[110]

Table 1.2 Gene expression investigations in adipose (A) and muscle (M) for normal and/or EMS/IR/ID/hyperinsulinemic horses

<table>
<thead>
<tr>
<th>Study</th>
<th>Tissue</th>
<th>Proteins/Genes Examined</th>
<th>Significant Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>deLaat 2015[115]</td>
<td>M</td>
<td>Glucose transporter (GLUT) 1, 4, 8, 12</td>
<td>Decreased GLUT1 in hyperinsulinemic horses</td>
</tr>
<tr>
<td>Morrison 2014[111]</td>
<td>M, A</td>
<td>Myostatin (MSTN), Activin receptor IIB (ActRIIB), Follistatin (FST), Perilipin (PLIN), MSTN, ActRIIB, FST, PLIN</td>
<td>MSTN and ActRIIB and their genes only present in muscle, Perilipin (gene and protein) only present in adipose. Follistatin gene in many tissues. All normal horses.</td>
</tr>
<tr>
<td>Burns 2010[110]</td>
<td>A</td>
<td>Tumor necrosis factor- alpa (TNF-alpha), Interleukin (IL) 1beta (IL-1beta), IL-6, Plasminogen activator inhibitor1 (PAI-1), Monocyte Chemoattractant</td>
<td>IL-1beta and IL-6 higher in nuchal ligament adipose depot vs. other depots, no difference between SI and IR horses gene expression</td>
</tr>
</tbody>
</table>
Table 1.2 (cont’d)

<table>
<thead>
<tr>
<th>Author</th>
<th>Type</th>
<th>Markers</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bruynsteen 2013[96]</td>
<td>A</td>
<td>Protein-1, leptin, chemokine ligand 5, interleukin 1β, interleukin 6, interleukin 10, adiponectin, matrix metalloproteinase 2, and superoxide dismutase 2</td>
<td>leptin, chemokine ligand 5, interleukin 10, interleukin 1β, adiponectin, and matrix metalloproteinase 2 differed across depots</td>
</tr>
<tr>
<td>Banse 2016[112]</td>
<td>M</td>
<td>TNF-alpha</td>
<td>TNF alpha lower in obese hyperinsulinemic horses</td>
</tr>
<tr>
<td>Banse 2015[113]</td>
<td>M</td>
<td>Markers of oxidative stress (oxidative damage, mitochondrial function, and antioxidant capacity)</td>
<td>estrogen-related receptor alpha, ERRα increased with increasing BCS</td>
</tr>
<tr>
<td>Basinska 2015[114]</td>
<td>A</td>
<td>IL-6, TNF-alpha</td>
<td>IL-6 increased in horses with EMS</td>
</tr>
<tr>
<td>Selim 2015[60]</td>
<td>A</td>
<td>Insulin receptor, retinol binding protein 4, leptin, and monocyte chemoattractant protein-1, adiponectin (ADIPOQ), adiponectin receptor 1 and stearoyl-CoA desaturase (SCD)</td>
<td>Down-regulation of insulin receptor, retinol binding protein 4, leptin, and monocyte chemoattractant protein-1, and up-regulation of adiponectin (ADIPOQ), adiponectin receptor 1 and stearoyl-CoA desaturase (SCD) in both normal and EMS horses during the grazing season</td>
</tr>
</tbody>
</table>

RNA Seq: Current Equine Research and the Potential Impact of the Technique

RNA Seq analysis is an unbiased approach to gene expression that can help identify pathways of expression within the tissues. It is unbiased as it reports the entirety of the mRNA present at that time, as opposed to a few of interest selected a priori. In the horse, RNA Seq techniques to date have been performed in several tissues,[116] such as the skeletal muscle of race horses looking for genes related to exercise,[117,118] laminar tissue looking for genes related to inflammation and matrix stability,[119] and trophoblasts investigating development.[120] While there are known genes that are associated with increased insulin sensitivity in muscle and brown adipose tissue (GLUT4, myostatin, irisin, some peroxisome
proliferator-activated receptors), there are also those genes traditionally associated with increased insulin resistance (HIF1 alpha, TNF alpha, IL-1, IL-6).[93] Using RNA-seq to quantify gene expression allowing for differential gene expression and pathway analysis across all genes in skeletal muscle and adipose tissue offers the potential of moving beyond these commonly investigated genes to identify novel genes that may play important roles in the pathophysiology of EMS.[121-123]

**Hypotheses and Aims**

Central hypothesis: Breed differences in metabolic phenotype will be reflected in breed differences in insulin dynamics, lipid metabolism, and the histologic and metabolic phenotype of skeletal muscle and adipose tissue.

**Aim 1: To compare whole body measures of metabolism among five breeds of horses/ponies (Quarter Horses, Arabians, Morgans, Welsh Ponies, and Thoroughbreds)**

The hypothesis is that QH will demonstrate a healthier metabolic phenotype as reflected by high insulin sensitivity, a low acute insulin response, and glucose-mediated glucose disposal, a higher apparent rate of fatty acid oxidation, lower fasting serum triglyceride (TG) and leptin concentrations and higher HMW adiponectin when compared to other breeds. Thoroughbreds will be similar to QH. Morgan horses will be intermediate between QH and Arabians/WP in relation to these metabolic characteristics. This aim will be accomplished via:

a) Minimal model analysis of an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT), including the acute insulin response to glucose (AIRg), insulin sensitivity (SI), and glucose dispersal
b) Evaluation of an Arginine Stimulation Test (AST) for assessment of an acute insulin response

c) Assessment of insulin and glucose responses to an Oral Sugar Test (OST), including peak insulin and glucose concentrations and areas under the concentration by time curves as well as trajectories for glucose and insulin

d) Measurement of fasting serum TG, leptin, and HMW adiponectin concentrations

**Aim 2: To compare skeletal muscle and adipose tissue cellular phenotype among the breeds** The hypothesis is that QH will demonstrate a higher proportion and greater diameter of skeletal muscle Type 2b fibers, and lower adipocyte diameter and volume, characteristics which are associated with higher insulin sensitivity when compared to other breeds. Thoroughbreds will be similar to QH. Morgans will be intermediate between QH and Arabians/WP in relation to these phenotypic characteristics. This aim will be accomplished via:

a) Measurement of skeletal muscle fiber type diameter, area, and proportions

b) Measurement of adipocyte diameter and volume in subcutaneous (tailhead region) adipose tissue

**Aim 3: To compare skeletal muscle and adipose tissue gene expression among four breeds** The hypothesis is that QH will demonstrate upregulation of gene expression pathways linked to enhanced insulin signaling and brown adipose tissue-like adipose tissue phenotype when compared to other breeds. Morgan horses will be intermediate between QH and Arabians/WP in relation to the expression of genes in these pathways. This aim will be accomplished via:

a) Characterization of the transcriptome of skeletal muscle and adipose tissue, collected in Aim 2, via RNA Seq.
Significance of the work: Lack of information regarding the pathophysiology of EMS limits the ability to predict disease risk associated with EMS and hinders the identification of horses and ponies that will benefit from preventative measures and therapeutic intervention prior to disease onset. Understanding breed differences in skeletal muscle and adipose tissue as it relates to insulin sensitivity and other metabolic traits will greatly advance understanding of the molecular pathophysiology of EMS and laminitis susceptibility.
2. Breed Differences in Insulin and Glucose Dynamics during Challenge Testing

Evaluation of an arginine stimulation test for assessment of acute insulin response in adult horses

Summary

Background: Insulin dysregulation (ID) is a feature of equine metabolic syndrome, with acute hyperinsulinemia being associated with causing laminitis. Testing methods for quantitative determination of the acute insulin response (AIR) are needed to identify “at risk” individuals. The arginine stimulation test (AST) has not been evaluated in adult horses.

Objectives: To (1) determine the acute insulin response to different dosages of intravenous (IV) arginine (AIRarg), (2) evaluate the repeatability of AIRarg, (3) compare the AIRarg to the acute insulin response to IV glucose (AIRglu), and (4) evaluate the association between AIRarg and minimal model insulin sensitivity (SI) derived from an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT).

Study Design: In vivo experiment

Methods: The AST was conducted in 6 Thoroughbred horses at 70 mg/kg and 100 mg/kg IV. The 70 mg/kg dose was also administered to 21 Arabians. Repeatability was assessed in 10 horses. An FSIGTT was performed at least 48 hours apart from AST testing to determine the response to glucose and SI. Statistics used included: repeated measures ANOVA, Bland-Altman analysis, and Spearman correlations. Significance was set at P<0.05.
Results: One minute post-arginine administration, mean (± SD) insulin concentrations increased from a baseline value of 7.5 ± 6 μIU/ml to 30.9 ± 11 μIU/ml, and remained significantly greater than baseline for 15 minutes. The AIRarg was repeatable and did not differ between doses of arginine. There was a strong association (\(rho= 0.69, P<0.001\)) between AIRarg and AIRglu, but not SI.

Main Limitations: This study was not designed to determine a threshold for insulin concentration to identify adult horses with insulin dysregulation.

Conclusions: Intravenous arginine testing in adult horses elicits a significant acute insulin response that is sustained for at least 15 minutes post administration.

Introduction

Hyperinsulinemia (basal) and/or exaggerated insulin response to oral glucose administration or feeding is a feature of the equine metabolic syndrome. [6,22] The reported clinical association between hyperinsulinemia and incident laminitis, as well as the experimental induction of laminitis following 48 hours of euglycemic-hyperinsulinemia [26,27] emphasize the need for practical and reliable tests for evaluation of insulin dynamics in horses. In clinical practice, insulin dysregulation has been evaluated by measurement of basal ("fasting") insulin concentrations or the insulin response to IV or oral glucose challenge (among other tests). However, few studies have evaluated the acute insulin response (AIR) in the adult horse[28], and it has not been assessed in any of the currently described field based testing protocols (such as the oral sugar test (OST), in feed glucose test, or the 2-step insulin response).[1,11,58,124]

In humans – studies often use glucose, arginine and/or glucagon for stimulation testing of beta-cell function.[125,126] Arginine directly depolarizes the beta-cells of the pancreas to
release insulin [127] and is thought to be superior to glucose stimulation for estimating the duration of the presence of disease (type 2 diabetes) and beta cell reserve in humans.[128] The AST has proved useful in assessing the AIR in multiple species (humans, cats, camelids, foals)[71,125,129-133] but has not been tested in adult horses. Therefore, the objectives of this study were: (1) to compare two doses of arginine that have been used in various species (70 mg/kg bwt) and 100 mg/kg [71,132,133]); (2) to determine the repeatability of the AIR to IV arginine; (3) to compare an arginine versus a glucose stimulus for the determination of the AIR; and (4) to assess the correlation between the AIRarg and minimal model sensitivity (SI) derived from a FSIGTT.

**Methods**

**Subjects**

Six adult Thoroughbreds (5 mares, 1 gelding, age range 8-16 years) and twenty-one adult Arabian horses (14 mares, 7 geldings, age range 3-23 years) were used in this study. All horses were kept in individual stalls during the testing periods and hay and grain was removed at 10 PM the night before each testing period. Jugular catheters were placed approximately 45 minutes prior to testing and all horses were accustomed to having jugulars catheters placed. All horses were owned by Michigan State University. All experiments were approved by the university’s Institutional Animal Care and Use Committee.

**Study Design**

All testing occurred during late spring or summer. The six Thoroughbreds were used for the AST dose comparison. Both the AST and FSIGTT were performed on the twenty-one adult Arabian horses with least 48 hours between the two tests. Five of the Arabians, in addition to
five of the Thoroughbreds from the dose comparison study, were included in the repeatability part of the testing. All 10 horses had a second AST performed at least 24 hours after the previous test.

*Arginine Stimulation Test (AST)*

For the dose comparison, baseline blood samples were obtained and horses were randomly assigned to receive either a 70 mg/kg bwt or a 100 mg/kg bwt intravenous (IV) bolus of arginine HCl (200 mg/ml) given over 30 seconds in a cross-over design. Subsequent blood samples were obtained at 1, 2, 3, 4, 5, 7, 10, and 15 minutes. After at least 24 hours, the AST was repeated at the other dose. The lower dose (70 mg/kg) was selected to be used for all other testing. The 1, 2, 3, 4, 5, 7, 10, and 15 minute sampling time points were utilized for all tests.

*Frequently Sampled Intravenous Glucose Tolerance Test (FSIGTT)*

Baseline blood samples were obtained followed by a 300 mg/kg bwt IV bolus of 50% dextrose. Insulin (Humulin R, 20 IU/kg) was given at 20 minutes post dextrose via an IV bolus. Blood samples for insulin and glucose evaluation were collected at 1, 2, 3, 4, 5, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, 180, 210, and 240 minutes post dextrose administration. The first 19 minutes of this test were considered an assessment of the glucose challenge’s effects on insulin response (AIRglu).

*Insulin and Glucose Determination*

Blood was collected into tubes without additives for insulin and into tubes with EDTA for glucose (dose comparison part of the study only). Glucose was only analyzed in the initial
dose response part of the study (tubes centrifuged and plasma harvested within 30 minutes to 1 hour of collection) for the AST; as glucose concentration did not differ over time in that test, it was not measured for the remainder of the study. All samples were centrifuged at 2000 x g for 15 minutes, with serum and plasma harvested and subsequently stored at -80°C. A commercially available RIA kit that has been previously validated for horses in our laboratory and by others [28, 65] was used for duplicate analysis of serum immunoreactive insulin. The glucose oxidase method [27] was used to analyze plasma glucose concentrations.

Calculations and Statistical Analyses

The Shapiro-Wilks test was used to assess normality. Blood insulin concentrations were analyzed for evaluation of the AIR [134], peak concentration, and time to peak concentration. Specifically, AIRarg and AIRglu were determined by taking the difference between the baseline insulin concentration and the mean of the three highest insulin readings from samples obtained at 2, 3, 4 and 5 min after arginine or glucose administration. Minimal model analysis of glucose and insulin data from the FSIGTT was performed to obtain SI using a dedicated program [8, 125, 135, 136]. Horses with an SI ≥ 1 were insulin sensitive (IS) and < 1 were considered insulin resistant (IR). One way repeated measures ANOVA with Bonferroni correction was used to evaluate the concentrations of insulin and glucose (dose comparison only) post-arginine administration to baseline insulin concentrations for an individual horse. A paired t-test was used to compare between AIRarg for two different doses of arginine. Repeatability of the AST was evaluated by Bland-Altman analysis. Spearman Correlation Coefficients were used to determine associations between several indices derived from the AST and FSIGTT, including: insulin concentrations at 2 and 5 minutes (AST$_{ins \ 2 \ min}$, FSIGTT$_{ins \ 2 \ min}$, AST$_{ins \ 5 \ min}$ and FSIGTT$_{ins}$
5 min), the overall highest peak insulin concentration from 0-15 minutes in the AST (AST$_{\text{peak}}$) and from 0-19 minutes in the FSIGTT (FSIGTT$_{\text{peak}}$), AIRarg vs. AIRglu and the AIRarg vs.SI. All statistics were performed with dedicated software. Data are reported as means ± SD unless otherwise stated. Significance was set at P<0.05.

**Results**

*Dose Comparison*

In response to a 70 mg/kg IV dose of arginine, mean insulin concentrations increased from a baseline value of 11 ± 3 μIU/ml to 25 ± 13 μIU/ml by one minute post arginine administration and remained significantly greater than baseline, with a mean of 23 ± 3 μIU/ml, at all time points measured during the first 7 minutes post arginine administration (P≤0.005)(Figure 2.1). As there was no difference in the AIR between doses (P=0.6), the lower dose was used subsequently in comparing the AST to the FSIGTT in the Arabians.
Figure 2.1 Mean (± SD) insulin concentrations in response to two doses of arginine HCl (70 mg/kg bwt, black circles; and 100 mg/kg bwt blue squares) in 6 adult Thoroughbred horses. (* indicates significantly different (P < 0.05) from baseline (0 min)).
Repeatability of the AST

When assessing repeatability of the AST for AIRarg via a Bland-Altman plot, the responses of all of the horses were within the 95% confidence interval (Figure 2.2 B). The AIRarg CV (+/- standard deviation) was 20% (±11). Mean insulin concentrations (µIU/ml) (n=10 adult horses) over time for the initial and repeat AST are shown in Figure 2.2 Mean (±SD) insulin concentrations (µIU/ml) (N=10 adult horses) (A) over time for the initial (black circles) and repeat (green squares) AST. Bland-Altman plot (with 95% CI) (B) of repeatability of the AIRarg in an AST performed in 5 adult Thoroughbreds and 5 adult Arabian horses. (* indicates significantly different (P < 0.05).

![Graph A](image1)

![Graph B](image2)

Figure 2.2 Mean (±SD) insulin concentrations (µIU/ml) (N=10 adult horses) (A) over time for the initial (black circles) and repeat (green squares) AST. Bland-Altman plot (with 95% CI) (B) of repeatability of the AIRarg in an AST performed in 5 adult Thoroughbreds and 5 adult Arabian horses. (* indicates significantly different (P < 0.05)
Performance of the AST and correspondence to indices from the FSIGTT in 21 adult horses

There were no significant differences in insulin concentration between IR (N=7) and IS (N=14) horses at any time point during the AST. One minute post-arginine administration, mean (± SD) insulin concentrations increased from a baseline value of 7.5 ± 6 μIU/ml to 30.9 ± 11 μIU/ml, and remained significantly greater than baseline for 15 minute post-administration (Figure 2.3). Time to peak insulin concentration was in the first 5 minutes post arginine administration in 19 of 21 horses (in 2 horses, insulin concentration peaked at 7 minutes post arginine administration). There were moderate to strong associations between indices from the AST and FSIGTT, specifically: AST_{ins 2 min} vs. FSIGTT_{ins 2 min} (\rho = 0.7), AST_{ins 5 min} vs. FSIGTT_{ins 5 min} (\rho = 0.64), AST_{peak} vs. FSIGTT_{peak} (\rho = 0.8) (Figure 2.4). When compared to the glucose challenge during the FSIGTT, the AST provided a similar assessment of the AIR in adult horses (\rho=0.69, P<0.0005) (Figure 2.5). When comparing AST_{arg} values with the SI derived from minimal model analysis of the FSIGTT, there was an inverse relationship but it was not a significant correlation (\rho=-0.25, P=0.2) (Figure 2.5). This was also true when comparing AIR_{glu} vs. SI (\rho = -0.39, P = 0.07) (Figure 2.5).
Figure 2.3 Mean insulin (+SD) concentration interval in response to arginine HCl (70 mg/kg) over time in 21 adult Arabian horses (A) (* indicates significantly different from the insulin concentration at 0 minutes; \( P < 0.05 \)), and in 7 insulin resistant (IR; blue squares) and 14 insulin sensitive (IS; black circles) adult Arabian horses (B).
Figure 2.4 Spearman correlations between indices from the AST and FSIGTT (N=21), specifically: AST$_{\text{ins 2 min}}$ vs. FSIGTT$_{\text{ins 2 min}}$ (A), AST$_{\text{ins 5 min}}$ vs. FSIGTT$_{\text{ins 5 min}}$ (B), AST$_{\text{peak}}$ vs. FSIGTT$_{\text{peak}}$(C).
Figure 2.5 Correlations between the AST and the FSIGTT (N=21), specifically: AIR\textsubscript{glu} versus AIR\textsubscript{arg} (A), SI versus AIR\textsubscript{arg} (B), and SI versus AIR\textsubscript{glu} (C).

Discussion

The IV administration of arginine (70 mg/kg) induced a measureable rise in insulin concentration in adult horses by one minute post arginine administration which was sustained for
the duration of sampling (15 min). The difference between peak and baseline insulin concentrations was greater in adult horses when compared to 2 or 10 day old pony foals (Peak-Baseline: 30.6 μIU/l ± 14.5 vs 9.8μμIU/ml ± 2.74 and 16.2 μIU/ml ± 3.17 respectively),[71] but was considerably lower than has been reported for cats (Peak insulin-Baseline insulin= 22.5 μIU/ml (horses) vs 76.4 μIU/ml (cats)).[131] In part, this discrepancy may be attributed to differences in age (pony foals vs. adults), and perhaps also the fact that the comparison is between ponies and horses. Notably, a 100 mg arginine/kg bwt dose has been used in humans, pony foals and cats, whereas the 70 mg/kg dose that has been employed in camelids was used in the study reported here.[131,132] The 70 mg/kg dose was considered preferable as there was not a significant difference in the response from the 100mg/kg dose, and the smaller dose has the advantage of cost savings and is easier and faster to administer as an IV bolus. Whereas previous studies have not included direct comparisons between doses, the human dose of arginine has been decreasing over time, from 500 mg/kg bwt initially,[137] to 150 mg/kg bwt,[138] and to 100 mg/kg.[126] In one study using cats, the AUC for insulin increased with increasing arginine dose but plateaued at 0.1 g/kg dose.[139] As the study used a 0.05 g/kg dose and a 0.1 g/kg dose, we are unable to assess whether the insulin response may have plateaued sooner (for example, at a 0.07 g/kg, the dose used in the present study). Although differences in insulin concentration between IR and IS horses during the AST have been included, overall power to determine differences between the groups was not sufficient as this was not the focus or design of the study. In foals, time to peak insulin concentrations occurred from 5-15 min,[71] whereas, in the adult horses of the study reported here, peak concentrations were attained in the first 5 min, a result more similar to that found in cats and humans.[125,131]
The AST performed in adult horses in this study showed a repeatable AIR$_{arg}$, which was expected as it is reported to be a highly repeatable test in humans.[125] This test is less repeatable than the AIR$_{glu}$ from the FSIGTT (CV% 11.7 +/- 6.5)[65] or the CGITT when evaluating insulin AUC (CV% 7.7).[59] The AST AIR$_{arg}$ appears to be more repeatable than the insulin AUC during the OST (CV% 29 +/- 11),[62] or of the insulin responses to the OGTT.[63] Although the OST does not specifically examine the AIR, it does assess the insulin response to an oral challenge. While a standard for repeatability has not been firmly established, ideally mean CV% would be closer to what is achieved during the FSIGTT and combined glucose and insulin tolerance test (CGIT).

The AIR$_{arg}$ had a strong correlation to the AIR$_{glu}$ from minimal model analysis. The AST’s strong agreement to the AIR$_{glu}$ is of greater interest as recently AIR$_{glu}$ has been suggested as a stronger predictor of clinical susceptibility to EMS than SI.[29] [140]. In pony foals, the peak-baseline difference in insulin response and the AUC for insulin was lower after arginine administration versus IV glucose, but direct correlations between the tests were not examined.[71] This finding is similar to what is reported in this study whereby the AIR$_{glu}$ exceeded the AIR$_{arg}$ in twelve horses and was very similar in five more and potentially indicates a species difference from cats where the AUC for insulin in response to arginine was greater than that of glucose.[131,139]

Neither the AIR$_{arg}$ nor the AIR$_{glu}$ had a significant inverse correlation to SI from minimal model analysis. This is somewhat surprising given that insulin secretion secondary to arginine administration was found to be inversely correlated with insulin sensitivity in humans[141] [140]. However, in one human study, only subjects with a baseline fasting glucose in the highest quartile of the normal range demonstrated this inverse association between AIR$_{arg}$ and insulin.
sensitivity as determined by a euglycemic-hyperinsulinemic clamp (EHC) method.[141] The results of this study also differ from previous studies in horses in which the inverse association between $\text{AIR}_{\text{glu}}$ and SI was significant.[3,142-145] Adrenaline has been shown to decrease insulin secretion,[146] and it is possible that catheter placement 45 minutes in advance of the test was an inadequate period of time to allow for adrenaline dispersal. This seems unlikely however as the horses were accustomed to placement of catheters, a procedure undertaken using a local skin desensitization using lidocaine and with minimal restraint required. What seems more likely is when examining the relationship between $\text{AIR}_{\text{glu}}$ and SI, horse number 31 was an influential point, and if removed, the relationship did gain significance. This horse did not similarly affect the relationship between $\text{AIR}_{\text{arg}}$ and SI however. Others have called into question whether IV testing should still serve as a reference test for determining IR vs IS horses,[29,54] suggesting an oral challenge test may have produced results that categorized this horse as IR rather than IS. This study did not see insulin concentration differences between IS and IR animals. However, this study was only designed to achieve the power needed to determine if there was a significant increase in insulin from baseline concentrations after arginine administration. Post-hoc calculated power to be able to determine a significant difference between these two groups at the 2 min timepoint was 23%, with 38 horses required in each group to achieve an 80% power.[147]

This study examined the acute insulin response to arginine in adult Thoroughbred and Arabian horses over a range of insulin sensitivities. The AST elicited a significant increase in serum insulin concentrations in a short time with an acceptable level of repeatability. The strong correlation between $\text{AIR}_{\text{arg}}$ and $\text{AIR}_{\text{glu}}$ provides justification for future studies to investigate the utility of the AST for evaluation of EMS. Further studies to evaluate breed variation in insulin response during the AST are also warranted.
Evaluation of a modified oral sugar test for dynamic assessment of insulin response and sensitivity in horses

Summary

**Background:** Veterinarians have identified equine metabolic syndrome (EMS) as the most common cause of laminitis within equine practice, and there is an established association between hyperinsulinemia (a clinical sign of EMS) and laminitis. Detecting abnormalities in insulin dynamics is therefore critical to the identification of individuals at risk for laminitis.

**Objectives:** To determine the optimum sampling protocol and clinical thresholds for insulin and/or glucose in an oral sugar test (OST) in identifying horses with insulin dysregulation (ID) across a
range of insulin sensitivities. To compare an OST to the frequently sampled intravenous glucose tolerance test (FSIGTT) and several calculated indices for assessment of insulin response.

**Study Design:** Analytic randomized prospective crossover study

**Methods:** A modified OST (0.25 ml/kg light corn syrup syrup) and an FSIGTT was performed on eighty-two horses/ponies of a range of insulin sensitivities. Statistics included: repeated measures ANOVA, ROC curve analysis, and Spearman correlations. Significance was set at P<0.05.

**Results:** A single time point blood sample for measurement of the insulin concentration obtained at 60, 75, 90, or 120 minutes with an insulin concentration of ≥22.8, 18.7, 30.2 or 26.3 µIU/mL at these time points, respectively, was indicative of ID, except in Morgans. Moderate correlations (ρ=-0.61 to -0.63, P<0.001) to insulin sensitivity (SI) derived from minimal model analysis of the FSIGTT and strong correlations (ρ=0.74 to 0.77, P<0.001) to AIRg were evident for area under the curve for insulin (AUCi), peak, and overall mean insulin. Weak correlations existed between glucose concentrations from the OST and SI and/or AIRg. All indices had no better than moderate correlation to SI (ρ<0.59) but had moderate to strong correlations to AIRg.

**Main Limitations:** In horses, breed appears to be a factor in insulin responses.

**Conclusions:** Determination of insulin concentration from a single blood sample obtained at 60, 75, 90, or 120 minutes during an OST that is greater than ≥22.8, 18.7, 30.2 or 26.3 µIU/mL respectively provides a reasonable diagnostic test for identifying ID, as defined by horses that are classified as insulin resistant based on an FSIGTT.
Introduction

Veterinarians have identified equine metabolic syndrome (EMS) as a major concern and the most common cause of laminitis within equine practice.[1-3] Insulin dysregulation (ID), a term that encompasses both the hyperinsulinemia and insulin resistance that occurs in EMS, has been associated with driving the development of laminitis in horses. More specifically, hyperinsulinemia has been reported to experimentally induce laminitis in otherwise healthy animals.[26,27,51] Detecting abnormalities in insulin dynamics is therefore critical to the identification of individuals at risk for laminitis. In veterinary practice, static fasting insulin is most commonly assessed.[1] However, clinical experience indicates low sensitivity of fasting insulin for the identification of equids with a history of EMS-associated laminitis. Currently, there are several dynamic intravenous and oral tests used to evaluate insulin and glucose dynamics, with the focus of these tests being further understanding of tissue level insulin sensitivity, the acute insulin response to glucose (AIRg), or both. In research settings, the frequently sampled intravenous glucose tolerance test (FSIGTT) is often used to assess both the AIRg and tissue level insulin sensitivity.[28,54,65] While the FSIGTT is a useful research tool, the time (>4 h), number of blood samples required and the related expense makes it impractical for routine diagnostic use.

Of more practical clinical use is an Oral Sugar Test (OST) that has recently been applied for the assessment of insulin response in horses and ponies.[54,64,148] The OST involves oral administration of corn syrup and the collection of only 1-2 blood samples. To date, however, there are no published data on the validity of an OST for assessment of insulin response over a wide range of known insulin sensitivities in a large number of equids of different breeds. A more rigorous evaluation of the test is needed to determine optimum sampling time points/interval(s)
and thresholds for ID during an OST, as well as to assess further the utility of calculated indices in approximating insulin sensitivity based on an oral glucose challenge.[57,149-151] Our objectives were to: 1) compare the results of an OST with parameters estimated by the FSIGTT; 2) use these data to determine the optimal sampling protocol and clinical thresholds of insulin and glucose for an OST; and 3) determine which, if any, mathematical indices derived from OST responses improve the correlation between this test and the FSIGTT. We hypothesized that a measurement or combination of measurements from an OST will be highly correlated to insulin sensitivity (SI) and acute insulin response to glucose (AIRg) as determined by minimal model analysis of an insulin-modified FSIGTT and, therefore, will provide a clinically useful diagnostic test for evaluation of insulin dynamics and for determining ID in horses.

**Materials and Methods**

**Horses**

Eighty-two horses (age range 3-25 years; 37 geldings and 45 mares) from five different breeds (22 Quarter Horses [QH], 21 Arabians, 21 Morgans, 6 Thoroughbreds [Tb], and 12 Welsh Ponies [WP]), representing a range of insulin sensitivities were utilized for this study. Horses were either institution or client owned. All protocols performed were approved by Michigan State University’s IACUC as well as the respective institution’s IACUC and/or a client consent form.

**Experimental Design**

All horses were sampled between May and the first week of August at one of the following locations: Manhattan, KS (QH), East Lansing, MI (Arabians and Tb), Storrs, CT (Morgans), Chazy, NY (Morgans), and Olive Branch, MS (WP). Horses were not in work during the testing
weeks and were maintained on their normal ration of predominantly grass hay (2-2.5% body weight). Horses were kept in stalls and food, but not water, was withheld from 10 PM the night before testing days. Horses were randomly allocated to undergo either the FSIGTT or OST in a randomized crossover design, with at least 24 hours (maximum 3 days) between tests. Feed was withheld during tests, with testing starting by 9:30 AM.

*Frequently Sampled Insulin-Modified Intravenous Glucose Tolerance Test (FSIGTT)*

To determine insulin sensitivity, the FSIGTT [9; 10] first described by Hoffman et al.[144] followed by Minimal Model Analysis (MinMod Millennium V6.0) was used. A jugular catheter was placed at least 30 minutes prior to the start of the test. A baseline blood sample for insulin and glucose measurements was collected (0 minutes), followed by administration of a 300 mg/kg intravenous (IV) dextrose dose, with a 20 µIU/kg IV insulin (Humulin R) dose given 20 minutes later. Blood samples (for insulin and glucose measurements) were collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, 180, 210, and 240 minutes after dextrose had been administered.

*Oral Sugar Test (OST)*

For the OST, corn syrup (Karo Light Corn Syrup (1 pint size) a (heretofore referred to as corn syrup) was used in all tests. Corn syrup was administered at a higher dose than previously reported (0.25 ml/kg vs 0.15 ml/kg).[11] This choice of dose was seen as a good compromise between eliciting an insulin response in IS horses, (not always possible with the lower dose), and maintaining ease of administration with a manageable amount of corn syrup.
A jugular catheter was placed a minimum of 30 minutes before testing. A 0.25 ml/kg dose of corn syrup\(^a\) (~125g of sugars for a 500-kg horse) was administered by mouth via a dosing syringe after baseline blood samples (for insulin and glucose measurement) had been obtained. Subsequent blood sampling (for insulin and glucose measurements) took place at 15, 30, 60, 75, 90, 120, 150 and 180 min post corn syrup administration.

Biochemical and Hormonal Analysis

Blood was collected into tubes with no additives (insulin) or into Na heparin or NaF/K-oxalate (glucose) tubes. Blood was centrifuged on site and serum or plasma pipetted and stored at -80ºC until analysis. Glucose concentrations were determined via the glucose oxidase method (2300 STAT)\(^b\).[152] Insulin concentrations were determined via a radioimmunoassay previously validated for the horse (Coat-A-Count RIA)\(^f\).[14-16]

Glucose and Insulin Dynamics

Minimal model analysis (MinMod Millennium V6.0 and WinSAAM; http://www.winsam.org) was used to analyze the glucose and insulin data derived from testing in each horse, similar to previous reports. [28,57] Briefly, these analyses yielded estimates of SI (min\(^{-1}\)/mU/L; insulin-mediated glucose disposal) and AIRg ([mU/L]*min; a measure of the degree of insulin secretory response to glucose), glucose-mediated glucose disposal (Sg , min-1), disposition index (DI, DI = SI x AIRg; this describes the pancreatic beta-cell response), in addition to baseline values of insulin (Ib) and glucose (Gb). For further analyses, horses were classified as either insulin sensitive (IS; SI \(\geq\) 1) or insulin resistant (IR; SI \(\leq\) 0.99).
Calculated Indices

In several species including horses, a number of indices derived from oral glucose challenges have been used to best approximate insulin sensitivity. In this study, three such indices, the MATSUDA, SIisOGTT, and Avignon indices (Table 2.1) were calculated from samples derived from the modified OST for all horses.[57,150,151]

Table 2.1 Description of calculated indices from the OST

<table>
<thead>
<tr>
<th>Index</th>
<th>Equation using OST data</th>
<th>Min. model correlate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matsuda</td>
<td>10,000/sq root of ([(\text{fasting glucose} \times \text{fasting insulin}) \times (\text{mean glucose}<em>{OST} \times \text{mean insulin}</em>{OST})])</td>
<td>Insulin sensitivity</td>
</tr>
<tr>
<td>SIisOGTT</td>
<td>1/ [\log (\text{sum glucose}<em>{30+60+90+120+150+180}) + \log (\text{sum insulin}</em>{30+60+90+120+150+180})]</td>
<td>Insulin sensitivity</td>
</tr>
<tr>
<td>Avignon</td>
<td>[(0.137 \times \text{Sib}) + \text{si2h})/2</td>
<td>Insulin sensitivity</td>
</tr>
</tbody>
</table>

where Sib = 108/(fasting insulin \times \text{fasting glucose}); and Si2h = 108/(\text{insulin}_{120} \times \text{glucose}_{120})
Statistics

A repeated measures ANOVA was performed to evaluate OST insulin and glucose data over time between IS and IR horses. Significance was set at P < 0.05. ROC curve analysis was performed on random samplings of the data set to obtain thresholds of insulin that optimize sensitivity and specificity. This ROC analysis was bootstrapped 1000 times in a non-stratified manner (as number of cases did not match controls) using the pROC package to obtain confidence intervals around the ROC curve. Comparisons between OST outcome measures (area under the curve [AUC], peak concentrations, and time to peak concentrations for both insulin and glucose) to SI and AIRg as well as calculated indices from the FSIGTT were also made with Spearman Correlations (significant at P< 0.05). Statistics were carried out using dedicated software.\textsuperscript{g,h}

Results

The FSIGTT and OST procedures were successfully applied in 82 horses from 5 different breeds.

Insulin Sensitivity

Minimal Model analysis of the glucose and insulin data from the FSIGTT completed for all horses demonstrates the range of values within this cohort (Table 2.2). Based on SI criteria (SI $\leq 1$), there were 25 IR and 57 IS horses in this cohort.

Table 2.2 Data are median (interquartile range) values from a minimal model analysis of the FSIGTT of 90 adult horses. AIRg = acute insulin response to glucose, DI = disposition index, SI = insulin sensitivity, Sg = glucose dispersal outside of insulin effect.
Optimization of the OST for Sampling Time Points and Thresholds

Insulin and glucose concentrations for all horses over time during the OST were determined (Figure 2.6). No significant differences in glucose concentrations were detected between the IR and IS groups at any time points. Significant elevations in insulin concentrations were evident from 60 min until 120 minutes post corn syrup administration in the IR group as compared to the IS group. Differences of insulin concentrations between these time points were not seen within the IR group (Figure 2.6). The insulin thresholds which optimized sensitivity and specificity for diagnosing ID in the ROC curve analysis at the 60, 75, 90 and 120 min time points were ≥22.8, 18.7, 30.2 and 26.3 μIU/mL respectively (Table 2.3).

The sensitivities, specificities, positive (PPV) and negative (NPV) predictive values of certain insulin concentrations for these insulin thresholds are described in Table 2.3. These time points had median sensitivities of 57.69%, 73.68%, 57.69%, and 54.11% respectively (Table 2.3). The 75 and 90 min OST insulin concentrations were chosen as representative time points for displaying the ROC curves (Figure 2.7) because of a combination of their optimized sensitivity, number of false positives, PPV, and NPV.

<table>
<thead>
<tr>
<th>Horses (N=90)</th>
<th>AIRg</th>
<th>DI</th>
<th>SI</th>
<th>Sg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mU·min·L⁻¹</td>
<td>SI x AIRg</td>
<td>× 10⁻⁴ L·min⁻¹·mU⁻¹</td>
<td>× 10⁻²/min</td>
</tr>
<tr>
<td>151.8</td>
<td>(94.2-242.1)</td>
<td>276.3</td>
<td>(151.2-476.9)</td>
<td>2.2</td>
</tr>
<tr>
<td>2.2</td>
<td>(0.7-3.9)</td>
<td>1.5</td>
<td>(1.2-1.8)</td>
<td></td>
</tr>
</tbody>
</table>
A)  

B)  

Figure 2.6 Mean insulin (µIU/mL) (A) and glucose (mg/dL) (B) concentrations ± standard deviation over time for the OST (N=82). IS = insulin sensitive, IR= insulin resistant. *= significant difference between IS and IR horses at this time point.

Morgans were overrepresented when assessing false negatives using the respective insulin concentration thresholds from the OST from the 60, 75, 90 and 120 minute time points to suggest ID. Morgans represented 8 of 11, 6 of 7, 9 of 11 and 9 of 12 of the false negatives among those time points respectively. When the Morgan horses were removed from the data, the median
sensitivity of the OST between the 60 and 120 minute time points increased as much as 20% (Table 2.3).

Table 2.3 Median sensitivities (se.) and specificities (sp.) and their 95% confidence intervals (low and high), as well as positive (PPV) and negative (NPV) predictive values for all horses (N=82 top) or all horses except Morgans (N=62, bottom) for different timepoints and insulin thresholds during the OST.

<table>
<thead>
<tr>
<th>All horses (N=82)</th>
<th>Time</th>
<th>Insulin</th>
<th>Sp low</th>
<th>Sp median</th>
<th>Sp high</th>
<th>Se low</th>
<th>Se median</th>
<th>Se high</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
<td>22.85</td>
<td>80.39</td>
<td>89.66</td>
<td>96.49</td>
<td>37.92</td>
<td>57.69</td>
<td>76.19</td>
<td>71.4</td>
<td>81.9</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>18.65</td>
<td>67.92</td>
<td>78.57</td>
<td>88.89</td>
<td>54.17</td>
<td>73.68</td>
<td>89.66</td>
<td>61</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>30.2</td>
<td>76.36</td>
<td>86.21</td>
<td>94.44</td>
<td>37.04</td>
<td>57.69</td>
<td>75</td>
<td>65</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>26.35</td>
<td>80.77</td>
<td>89.66</td>
<td>96.49</td>
<td>34.62</td>
<td>54.11</td>
<td>73.69</td>
<td>70</td>
<td>80.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No Morgans (N=62)</th>
<th>Time</th>
<th>Insulin</th>
<th>Sp low</th>
<th>Sp median</th>
<th>Sp high</th>
<th>Se low</th>
<th>Se median</th>
<th>Se high</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
<td>22.85</td>
<td>81.82</td>
<td>91.3</td>
<td>97.92</td>
<td>60</td>
<td>82.35</td>
<td>100</td>
<td>72.2</td>
<td>93.2</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>18.65</td>
<td>65</td>
<td>78.05</td>
<td>89.13</td>
<td>79.16</td>
<td>94.12</td>
<td>100</td>
<td>76</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>32.7</td>
<td>85.11</td>
<td>93.48</td>
<td>100</td>
<td>60</td>
<td>82.35</td>
<td>100</td>
<td>70</td>
<td>95</td>
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<tr>
<td></td>
<td>120</td>
<td>26.35</td>
<td>79.07</td>
<td>89.13</td>
<td>97.62</td>
<td>52.94</td>
<td>75</td>
<td>93.75</td>
<td>70.5</td>
<td>90.1</td>
</tr>
</tbody>
</table>
Figure 2.7 ROC curve of the median and 95% confidence intervals for sensitivity and specificity at 90 (A) and 75 minutes (B) during an OST (N=82).
Correlation of SI to AIRg

SI had only a moderate correlation to AIRg \( (\rho = 0.54, P < 0.0001) \) (Figure 2.8).

Figure 2.8 Spearman correlations between insulin sensitivity (SI) and the acute insulin response to glucose (AIRg) both calculated from a frequently sampled intravenous glucose tolerance test (FSIGTT) \((N=90)\).
Figure 2.9 Spearman correlations of insulin sensitivity (A) or AIRg (B) to area under the curve for insulin (AUCi) during an oral sugar test (OST). Spearman correlations of insulin sensitivity (C) or AIRg (D) to peak insulin concentrations during an OST. Spearman correlations of insulin sensitivity (E) or AIRg (F) to overall mean insulin concentrations (μIU/mL) in 82 adult horses during an OST (all P<0.001).

Correlation of OST Outcome Measures to SI and AIRg

Moderate mathematical correlations to SI were evident for AUCi (rho = -0.62, P<0.001), peak insulin (rho = -0.63, p <0.001), and overall mean insulin (rho = -0.61, P<0.001) (Figure
2.9), although determining a true clinical interpretation using the data would be difficult. Weak correlations existed between SI and all glucose measures from the OST (all with \( \rho < 0.31 \)). Strong correlations to AIRg were evident for AUCi (\( \rho = 0.74, P<0.001 \)), peak insulin (\( \rho = 0.77, P<0.001 \)), and overall mean insulin (\( \rho = 0.75 \)) (Figure 2.9). Weak correlations existed between AIRg and all glucose outcome measures from the OST (all with \( \rho < 0.07 \)).

**Correlation of Calculated Indices to Minimal Model Parameters**

All indices had no better than moderate correlation to SI (\( \rho < 0.59 \)) whereas there was a range of moderate to strong correlations to AIRg (Table 2.4). Although some indices were significantly related to DI and Sg, there were only weak to no correlations to the indices (Table 2.4). Whereas the MATSUDA was strongly correlated to AIRg (\( \rho = -0.72, P<0.001 \)), only a moderate correlation to AIRg was evident for the SlisOGTT (\( \rho = -0.69, P<0.001 \)) and the Avignon (\( \rho = -0.60, P<0.001 \)) (Figure 2.10).

Table 2.4 Spearmans’ \( \rho \) correlation coefficients between minimal model analyses and the calculated indices. * = significant at \( P < 0.05 \).

<table>
<thead>
<tr>
<th></th>
<th>MATSUDA</th>
<th>SlisOGTT</th>
<th>Avignon</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI</td>
<td>0.43*</td>
<td>0.6*</td>
<td>0.36</td>
</tr>
<tr>
<td>AIRg</td>
<td>-0.72*</td>
<td>-0.69*</td>
<td>-0.60*</td>
</tr>
<tr>
<td>DI</td>
<td>0.04</td>
<td>0.24*</td>
<td>0.03</td>
</tr>
<tr>
<td>Sg</td>
<td>0.24*</td>
<td>0.24*</td>
<td>0.25*</td>
</tr>
</tbody>
</table>
Figure 2.10 Spearman correlations of calculated indices from the OST to AIRg: MATSUDA (A), SIisOGTT (B) and Avignon (C) (all P < 0.001).
Discussion

During the modified OST, an insulin concentration of ≥22.8, 18.7, 30.2 or 26.3 μIU/mL at 60, 75, 90, or 120 minutes, respectively, was suggestive of ID in this cohort of animals with the exception of Morgans. Unlike previously published studies, glucose was not a good discriminator of IR vs IS horses/ponies.[11] The OST had strong correlations to the AIRg and moderate mathematical correlations to SI determined from an FSIGTT reference test. Calculated indices were not more strongly correlated to SI or AIRg from the reference test than peak insulin concentrations during an OST alone.

This is the first study to rigorously evaluate an optimal threshold insulin concentration for indicating ID based on an OST, although several groups have compared responses to the OST in a smaller number of horses to a reference test.[11,54] In a previous study,[11] an insulin concentration of greater than 60 μIU/mL was used as a threshold to indicate insulin resistance in the sample cohort. Application of the same criteria to the horses in this study would have resulted in misclassifying 16 IR horses as IS. The 7 horses that would have classified as being IR were properly classified (overall yielding a sensitivity of 19% and a specificity of 100%). As the OST is a screening test, ideally the test would tend to be less conservative so that “at-risk” horses would not be missed, suggesting that a lower threshold concentration of insulin (30.2 μIU/mL at 90 min for example) would be of more clinical value. As shown above, relying on a resting insulin concentration alone, all but one IR horse would have been classified as IS, demonstrating the need for dynamic testing clinically. Horses in this study had hay removed at 10 PM, having an approximately 10 hour fast. While Knowles et al.[62] find no effect of fasting or feeding on insulin concentrations during an OST, Bertin et al.[61] did, with horses having a lower insulin
response after a twelve hour fast. They advocated performing only a 3 hour fast, which may result in higher insulin responses invalidating our above recommendations for insulin thresholds.

Despite the administration of a higher dose of oral sugar (0.25 ml/kg vs 0.15 ml/kg), in the present study we did not detect a difference in glucose concentrations between the IR and IS groups as was seen in a previous study.[148] The lack of a difference in glucose concentrations between horses in the IS and IR groups in this study is also in contrast to the human literature, where glucose concentrations in response to an oral sugar challenge have been exclusively used to screen for metabolic syndrome or diabetes.[17; 18] This lack of a difference may be because the IS group in this study were from five different breeds. In the previous Schuver et al. study, the controls (IS horses) were exclusively Quarter Horses, and the cases were from an assortment of breeds.[11] As breed differences in insulin and glucose responses have now been reported, with Quarter Horses having the lowest glucose concentrations of all the breeds,[5] these breed differences may have affected their interpretation.[148] Another possible reason for this lack of a difference between IR and IS horses’ glucose concentrations could be a result of oral sugar dose size as the 0.25 ml/kg dose is not a large enough dose to cause a dramatic increase in total sugars. Recently, oral glucose tests have been performed with 1 g/kg glucose in order to determine ID with good repeatability,[29] which differed from the poor repeatability of the OST reported by Knowles et al. in which a lower dose of sugar was administered.[62] Our findings indicate that glucose concentrations during the OST should not be used to classify a horse as IR or IS.

The OST appears to provide several outcome measures which have strong correlations to the acute insulinemic response to glucose (AIRg) as determined by Minimal Model analysis of an insulin-modified FSIGTT and, therefore, does provide a clinically useful diagnostic test for
evaluation of insulin dynamics and ID in horses. The moderate correlations to insulin sensitivity (SI) likely wouldn’t be clinically useful to distinguish ID from IS horses given the distribution of the data, although a mathematical correlation exists. Interestingly as well, SI and AIRg only had a moderate correlation, emphasizing that an acute hyperinsulinemic response does not always go hand in hand with tissue level insulin resistance. Overall, the OST AUC insulin, peak insulin concentration and overall mean insulin concentration had better correlations to the AIRg than SI, suggesting that AIRg may be a more important in classifying horses as ID. As an oral trigger with resultant insulin spike, the OST challenge may more closely resemble the insulin response elicited by exposure to eating lush pasture.\cite{11,29} In this sense, the AIRg may prove to be a better predictor of susceptibility to laminitis than SI. The idea that an oral challenge test may better assess both insulin and incretin responses that may be responsible for the development of laminitis more than an intravenous based test has recently been examined.\cite{29} In that study, more horses were found to have high insulin responses after an oral versus an IV challenge test.\cite{29} This idea calls into question the typically held belief that intravenous tests such as the FSIGTT or the euglycemic-hyperinsulinemic clamp should be reference tests for classifying horses as ID. The mean and median AIRg of this study were lower than previously reported. Treiber et al. found an AIRg mean of 270, a median of 218, and a 2.5th to 97.3th percent reference interval of 67-805, but it wasn’t clear what dose of insulin was used in that study (it could have been higher than what was used here).\cite{153}

Similarly, the calculated indices, particularly the MATSUDA, had stronger associations with the AIRg than SI, which is notable as those indices are typically considered proxies for SI. This is consistent with the findings of Pratt-Phillips et al.\cite{57} in which a similarly moderate correlation between an oral glucose tolerance test and the SI as determined from minimal model
analysis of an FSIGTT was noted. Overall, use of the calculated indices, based on their correlations to the reference test, did not appear more beneficial than using peak insulin concentration alone. This is largely driven by the use of glucose concentrations in the calculation, as glucose was shown to be not significantly different in distinguishing between IS and IR animals.

Although the Minimal Model analysis of FSIGTT results allowed the horses in the present study to be classified as IS or IR, one limitation was the inability to follow the study cohorts over time to determine whether horses in either group developed laminitis. Additionally, in order to accumulate OST data for a large number of horses and several breeds, testing involved multiple sites/farms albeit the facilities studied had similar management, nutrition, and exercise plans. The decision to utilize a higher dose of corn syrup in this study when compared to earlier OST studies (0.25 ml/kg vs. 0.15 ml/kg PO), makes direct comparisons difficult. It should also be noted that insulin concentrations determined are assay dependent. Therefore, although the general patterns would be expected to stay the same, the thresholds recommended may alter based on the assay used. It is also worth noting that breed differences in insulin response to the oral sugar challenge were not examined in this study. However, our finding of decreased sensitivity in response to this test when performed in Morgans, suggest that breed differences to the OST is an area to be pursued in more depth.

The OST appears to offer a reasonable screening test for the hyperinsulinemia component of ID in horses and ponies. Our findings indicate that a single time point blood sample obtained at 60, 75, 90 or 120 minutes post oral administration of 0.25 ml/kg bwt Karo light corn syrup provides sufficient sampling and that an insulin concentration $\geq 22.8$, 18.7, 30.2 or 26.3 $\mu$IU/mL, respectively, from this sampling can be used to classify horses with ID.
Evaluation of equine breed specific insulin and glucose dynamics in response to a frequently sampled intravenous glucose tolerance test and a modified oral sugar test

Summary

Background: Veterinarians have identified equine metabolic syndrome (EMS) as the most common cause of laminitis within equine practice, with an association noted between certain breeds and laminitis development. Understanding breed specific differences in lipid metabolism, adipokines concentrations, as well as insulin and glucose dynamics are critical to the identification of individuals at risk for laminitis.

Objectives: To determine breed differences in lipid metabolism, adipokines, as well as insulin and glucose responses to the FSIGTT and OST in five breeds of horses with varying susceptibilities to insulin dysregulation (ID).
Study Design: Analytic randomized prospective crossover study

Methods: Eighty-two horses/ponies from five breeds (Quarter Horse, QH; Arabian; Morgan; Welsh Pony, WP; Thoroughbred, TB) were used in this study. Blood samples were collected for analysis of lipid metabolism and adipokines and a modified oral sugar test (OST; 0.25 ml/kg light corn syrup syrup) and an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT) were also performed. Eight additional WP had these same tests performed. Minimal model analyses of insulin sensitivity (SI), the acute insulin response to glucose (AIRg), diposition index (DI), and glucose mediated glucose disposal (Sg), as well as the lowest glucose value (Gmin) and the deflection of glucose below baseline (dGB) during the FSIGTT were assessed. Statistics included: multilevel regression analysis, trajectory assessment, a one-way ANOVA, the Kruskal-Wallis test, ROC curve analysis, and Spearman correlations. Significance set at P<0.05.

Results: Significant breed differences existed between different markers for lipid metabolism and adipokines. Breed differences were evident in the results of the FSIGTT with QH determined to have significantly higher SI than all other breeds, a lower AIRg than WPs and Arabians, and a higher DI than Morgans. The AIRg was significantly higher in Arabians than in Morgans with a lower Sg than in WP. Morgans had a significantly lower AIRg than WPs. Gmin was significantly different by breeds (P=0.003), with Arabians, Morgans, and WP having lower Gmins than TBs. dGb was significantly different by breed (P=0.004), with Morgans having a greater dGb than QHs. For the OST, different insulin thresholds for ID existed for each breed. OST glucose and insulin trajectories and area under the curve also differed significantly by breed (lowest in QH).

Main Limitations: None of the QH was determined to be insulin resistant. There were a smaller number of WPs and TBs for the OST compared to other breeds.
Conclusions: Breed differences exist in static markers of EMS as well as glucose and insulin dynamic measurements during the FSIGTT and OST. Insulin thresholds for diagnosing ID differ by breed. Insulin and glucose trajectories may be more revealing than single time point thresholds for identification of at risk individuals.

Introduction

Insulin dysregulation (ID), which encompasses the idea of abnormal insulin and glucose responses to intravenous and oral challenges, is of recent interest due to its proposed role in equine metabolic syndrome (EMS) and laminitis.[1] Anecdotally, certain breeds appear predisposed to EMS and laminitis (Morgans, Arabians, Tennessee Walking Horses, Andalusians, Icelandic Horses, Dutch Warmblood and ponies in general) while others do not (QH, Standardbreds, Thoroughbreds).[6,10,66,73-76] Static assessments of insulin and glucose have a poor sensitivity for diagnosing ID and/or susceptibility to EMS[6,73,154,155], prompting investigations into other EMS defining traits as well as dynamic challenge tests for clinical use.[11,29,64] Baseline, static markers for lipid metabolism and adipokines, such as triglycerides, non-esterified fatty acids, leptin and adiponectin, have been associated with metabolic health and disease in the horse and can be considered EMS defining traits.[6,37,46] Understanding how these traits relate to breed differences reflected in insulin and glucose responses to dynamic testing is important for early EMS diagnosis and appropriate intervention.

Breed differences in response to dynamic testing have been examined in several insulin sensitive (IS) and insulin resistant (IR) breeds, although comparisons between breed responses to both an intravenous and oral challenge are few in number. Currently, breed related differences in
insulin sensitivity, insulin responses to a glucose-containing meal, and an oral glucose tolerance test (OGTT) have been investigated in Standardbreds, Andalusians, and ponies.[72] Ponies and Andalusians had higher peak insulin responses as well as area under the curve (AUC) for insulin during the OGTT, and a lower insulin sensitivity (SI) compared to Standardbreds.[72] In another study with these same breeds fed either cereal- or fat-rich meals, SI was again reported to be lower in ponies and Andalusians than in Standardbreds. During a combined glucose and insulin tolerance test, a more IR breed (Icelandic horses) was compared to a typically IS one (Standardbreds), and a difference in glucose, but not insulin dynamics was detected.[59] The rate of decrease in plasma glucose concentration was slower in the Icelandic horses, and a period of hypoglycemia was observed in both breeds during the test.[59] Even among the more traditionally IS breeds, there appear to be differences in insulin and glucose dynamics.[4] In a study comparing indices from an insulin-modified FSIGTT in Standardbreds, QHs and TBs, a period of hypoglycemia was observed in QHs and TBs but not Standardbreds; a lower insulin-to-glucose ratio maintained by Standardbreds during the FSIGTT, suggested that this breed may demonstrate stricter regulation of glucose homeostasis. Therefore, the aim of this study was to determine breed differences in markers of lipid metabolism, adipokines, as well as insulin and glucose responses to the FSIGTT and OST in five breeds of horses with varying susceptibilities to insulin dysregulation (ID). We hypothesized that breed-based insulin and glucose responses to dynamic testing would be apparent, and that calculated variables between the intravenous and oral challenge will be correlated to each other and differ by breed, while also being correlated to other EMS defining traits.
Materials and Methods

Horses

Eighty-two horses (age range 3-25 years; 37 geldings and 45 mares) from five different breeds (22 Quarter Horses [QH], 21 Arabians, 21 Morgans, 6 Thoroughbreds [Tb], and 12 Welsh Ponies [WP]), were utilized for the OST/FSIGTT comparison part of the study. An additional 8 WP were also included in analysis of the breed differences between the variables from the FSIGTT alone. Horses were either institution or client owned. All protocols performed were approved by Michigan State University’s Institutional Animal Care and Use Committee (IACUC) as well as the respective institution’s IACUC and/or a client consent form.

Experimental Design

All horses were sampled between May and the first week of August at one of the following locations: Manhattan, KS (QH), East Lansing, MI (Arabians and TB), Storrs, CT (Morgans), Chazy, NY (Morgans), and Olive Branch, MS (WP) and Greasy Corner, AK (WP). Horses were not subjected to structured physical activity (work) during the testing weeks and were maintained on their normal ration of predominantly grass hay (2-2.5% body weight). Horses were randomly allocated to undergo either the FSIGTT first and then an OST second or vice versa with at least 24 hours (maximum 3 days) between tests. Horses were kept in stalls and hay but not water removed overnight (10 PM) before testing days. Testing started by 9:30 AM and hay was withheld during the test.
**Frequently Sampled Insulin-Modified Intravenous Glucose Tolerance Test (FSIGTT)**

To determine insulin sensitivity, the FSIGTT procedure[9; 10] first described by Hoffman et al.[144] followed by Minimal Model Analysis (MinMod Millennium V6.0)\(^a\) was used to analyze insulin and glucose responses. A jugular catheter was placed aseptically prior to the start of the test. A baseline blood sample for insulin and glucose measurements was collected (0 minutes), followed by administration of a 300 mg/kg intravenous (IV) dextrose dose, with a 20 \(\mu\)IU/kg IV insulin (Humulin R)\(^b\) dose given 20 minutes later. Blood samples (for insulin, and glucose measurements) were collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, 180, 210, and 240 minutes after dextrose had been administered.

**Oral Sugar Test (OST)**

For an OST, light corn syrup (Karo)\(^c\) was administered at a higher dose than previously reported (0.25 ml/kg vs 0.15 ml/kg[11]). This decision was based upon our laboratories prior findings that the 0.15 ml/kg dose did not elicit a measureable insulin response in very insulin sensitive animals (particularly QH), while still maintaining a protocol with a manageable volume of corn syrup.[11] A jugular catheter was placed aseptically before testing. A 0.25 ml/kg dose of light corn syrup (Karo; 1 pint size)\(^c\) (~125g of sugars for a 500-kg horse) was administered by mouth via a dosing syringe after baseline blood samples (for insulin and glucose measurement) had been obtained. Subsequently, blood samples (for insulin and glucose measurements) were collected at 15, 30, 60, 75, 90, 120, 150 and 180 minutes post light corn syrup administration. For the OST, based on a previous study performed by our group, horses were classified as ID if a single time point sample obtained at 60, 75, 90 or 120 minutes post oral administration of 0.25
ml/kg bwt Karo light corn syrup resulted in an insulin concentration $\geq 22.8, 18.7, 30.2$ or $26.3$ µIU/mL, respectively.[154]

**EMS Defining Traits of Lipid Metabolism and Adipokines**

Blood for adipokine measurements (leptin and High Molecular Weight (HMW) adiponectin), as well as non-esterified fatty acids (NEFA), and triglyceride (TG) concentrations was obtained at the 0 time point of whichever test (FSIGTT or OST) was administered first.

**Biochemical and Hormonal Analysis**

Blood was collected into tubes with no additives (insulin, NEFAs, HMW adiponectin, leptin, TGs) or into Na heparin or NaF/K-oxalate tubes (glucose). Blood was centrifuged on site and serum or plasma pipetted and stored at -80º C until analysis. Glucose concentrations were determined via the glucose oxidase method (2300 STAT)\(^d\).[152] Insulin concentrations were determined via a radioimmunoassay validated for the horse (Coat-A-Count RIA)\(^e\).[14-16] HMW adiponectin concentrations were assessed with an ELISA\(^f\),[45], TGs by enzymatic hydrolysis\(^g\), NEFAs with an enzymatic colorimetric assay\(^h\), and leptin concentration was determined using an RIA\(^i\).

**Glucose and Insulin Dynamics**

Minimal model analysis (MinMod Millennium V6.0 and WinSAAM; http://www.winsam.org) were used to analyze the glucose and insulin data derived from FSIGTT testing in each horse. These analyses yielded estimates of insulin sensitivity (SI (min−1/mU/L); insulin-mediated glucose disposal), the acute insulin response to glucose (AIRg ([mU/L]*min); a
measure of the degree of insulin secretory response to glucose), glucose-mediated glucose disposal (Sg min-1)), disposition index (DI, DI = SI x AIRg; this describes the pancreatic beta-cell response), in addition to baseline values of insulin (Ib) and glucose (Gb). For further analyses, horses were either classified as either insulin sensitive (IS; SI ≥ 1) or insulin resistant (IR; SI ≤ 0.99).

Additionally, glucose deflection below baseline was also examined during the FSIGTT. Parameters studied during this deflection included: Gb, baseline glucose, Gmin, the lowest glucose concentration below baseline, Tmin, the time point at Gmin, Ge, the glucose at 240 min (sampling endpoint), dGb, the percent deflection of glucose below Gb, calculated as (Gb-G min) x 100/Gb, dGe, the percent deflection of glucose below Ge, calculated as (Gmin-Ge) x 100/Ge, HAUC, area under the curve below baseline glucose and Ttmax, the time point where glucose deflection returned to baseline.[4] Horses that did not have glucose deflections below baseline were excluded from this part of the statistical analysis.

Statistics

The Shapiro-Wilks test was used to assess normality. A one-way ANOVA was used to assess breed differences in age, weight, body condition scores and area under the curve (AUC) for insulin. The Kruskal-Wallis test with Dunn’s multiple comparisons test was used to assess breed differences in the MinMod output, the deflection of glucose below baseline, and AUC for insulin in the OST. ROC analysis was performed on insulin values from various time points in individual breeds during the OST, similarly to Manfredi et al.[154] to determine breed specific insulin thresholds for diagnosing ID. Insulin and glucose trajectories were assessed with multivariate growth curve modeling (using the likelihood ratio test, with the Bonferonni-Holm
correction for multiple comparisons, as well as the Wald Chi square test). All statistics were carried out on dedicated software. Significance was set at \( P < 0.05 \).

**Results**

*Study Population*

There were 22 Quarter Horses (QH), 21 Arabians, 21 Morgans, 20 Welsh Ponies (WP), and six Thoroughbreds (TB) in which a FSIGTT was performed. An OST was performed in only 12 of the 20 WP. Breed specific median ages, weights, and body condition scores (BCS) (95% Confidence Interval; CI) are in Table 2.5. Age differed (\( P < 0.001 \)) among breeds, with all breeds being significantly younger than the TBs, while QH were significantly younger than the WP. Median bodyweight also significantly (\( P < 0.001 \)) differed among the breeds, with WP bodyweight lower than all other breeds, and Morgans weighing significantly less than TBs. Median BCS did not differ among breeds (\( P = 0.08 \)).

*Baseline and Peak Insulin and Glucose Concentrations During the FSIGTT*

Breed differences in insulin and glucose concentrations during the FSGITT are reported in Table 2.6. Baseline insulin in QHs was less than all other breeds (all \( P < 0.04 \)). QH had lower baseline glucose concentrations than Morgan (\( P = 0.04 \)) and TB (\( P = 0.02 \)). Arabians had lower baseline glucose concentration than TB (\( P = 0.03 \)). QHs had lower peak insulin concentrations than Arabians (\( P < 0.001 \)), WPs (\( P = 0.004 \)), and TBs (\( P = 0.01 \)). QHs and Arabians had lower peak glucose concentrations than Morgans (\( P < 0.001 \)) and WPs (\( P < 0.001 \)).
Table 2.5 Median (95% CI) for age, weight, and body condition score (BCS) of five breeds. Values within columns with different superscript letters indicate significant differences between those breeds at P < 0.05.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Age</th>
<th>Weight (kg)</th>
<th>BCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>QH</td>
<td>3&lt;sup&gt;b&lt;/sup&gt; (2-6)</td>
<td>429.1&lt;sup&gt;b&lt;/sup&gt; (395.5-486.4)</td>
<td>5.25 (5-6)</td>
</tr>
<tr>
<td>Arabians</td>
<td>3&lt;sup&gt;bc&lt;/sup&gt; (3-7)</td>
<td>464&lt;sup&gt;bc&lt;/sup&gt; (428-377)</td>
<td>6.5 (5.5-7.5)</td>
</tr>
<tr>
<td>Morgans</td>
<td>5&lt;sup&gt;bc&lt;/sup&gt; (2-7)</td>
<td>445&lt;sup&gt;b&lt;/sup&gt; (390.5-477)</td>
<td>5.5 (5-6)</td>
</tr>
<tr>
<td>WP</td>
<td>10&lt;sup&gt;c&lt;/sup&gt; (7-14)</td>
<td>299.5&lt;sup&gt;a&lt;/sup&gt; (285.5-321)</td>
<td>5.5 (5-6)</td>
</tr>
<tr>
<td>TB</td>
<td>14.5&lt;sup&gt;a&lt;/sup&gt; (11-23)</td>
<td>538.4&lt;sup&gt;c&lt;/sup&gt; (507.7-588.2)</td>
<td>5 (4.5-7.5)</td>
</tr>
</tbody>
</table>

Table 2.6 Median (95% CI) for five breeds for baseline and peak insulin (μIU/mL) and glucose (mg/dL). Significant differences (P < 0.05) indicated by different letters in the column.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Baseline insulin</th>
<th>Baseline Glucose</th>
<th>Peak Insulin</th>
<th>Peak Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>QH</td>
<td>0.55&lt;sup&gt;a&lt;/sup&gt; (0-2.2)</td>
<td>82.8&lt;sup&gt;a&lt;/sup&gt; (80-87.2)</td>
<td>116.1&lt;sup&gt;a&lt;/sup&gt; (98-139)</td>
<td>329.8&lt;sup&gt;a&lt;/sup&gt; (312.5-352.5)</td>
</tr>
<tr>
<td>Arabians</td>
<td>5.6&lt;sup&gt;b&lt;/sup&gt; (4.3-8)</td>
<td>83.4&lt;sup&gt;a&lt;/sup&gt; (80.7-87.1)</td>
<td>204.4&lt;sup&gt;b&lt;/sup&gt; (173.8-336.1)</td>
<td>333&lt;sup&gt;a&lt;/sup&gt; (305.5-346.5)</td>
</tr>
<tr>
<td>Morgans</td>
<td>2.8&lt;sup&gt;b&lt;/sup&gt; (1.5-6.1)</td>
<td>89.3&lt;sup&gt;b&lt;/sup&gt; (86.7-91.9)</td>
<td>151.4&lt;sup&gt;ab&lt;/sup&gt; (133-220.7)</td>
<td>380.5&lt;sup&gt;b&lt;/sup&gt; (291.1-644)</td>
</tr>
<tr>
<td>WP</td>
<td>3.4&lt;sup&gt;b&lt;/sup&gt; (2-5.5)</td>
<td>84.6&lt;sup&gt;ab&lt;/sup&gt; (81.0-89.0)</td>
<td>173.1&lt;sup&gt;b&lt;/sup&gt; (152.6-351.4)</td>
<td>388.8&lt;sup&gt;b&lt;/sup&gt; (367.5-409)</td>
</tr>
<tr>
<td>TB</td>
<td>6.35&lt;sup&gt;b&lt;/sup&gt; (3.7-13.4)</td>
<td>97.3&lt;sup&gt;b&lt;/sup&gt; (87.9-102.6)</td>
<td>195.4&lt;sup&gt;b&lt;/sup&gt; (161-268.1)</td>
<td>392.8&lt;sup&gt;ab&lt;/sup&gt; (320.4-437.3)</td>
</tr>
</tbody>
</table>

**Minimal Model Variables**

Breed specific medians and ranges for each minimal model parameter are included in Table 2.7. Breed was significantly different with regards to SI (P<0.001), with QH having a significantly
higher SI than all other breeds except TBs. Breed was significantly different with regards to AIRg (P<0.001), with QH having a lower AIRg than all other breeds except Morgans and TBs. Arabians had a significantly higher AIRg than Morgans. Morgans had a significantly lower AIRg than WPs. Breed was significantly different with regards to Sg (P=0.03), with Arabians having a lower Sg than WPs. Breed was significantly different with regards to DI (P=0.004), with QH having a higher DI than Morgans.

Glucose Excursion Below Baseline

Breed differences in the parameters used to describe the excursion below baseline in the FSIGTT are reported in Table 2.8. Of the 90 horses, there were 88 horses with at least one glucose value below baseline (hypos). The average time to Gmin was 213 minutes (range 70-240 minutes), with only 23 horses not having the lowest glucose concentration at either 210 or 240 minutes. Of those 23 horses, most reached the lowest glucose concentration at 180 minutes (15 of 23). Gmin was significantly different by breed (P=0.003), with Arabians, Morgans, and WP having lower Gmins than TBs. dGb was significantly different by breed (P=0.004), with Morgans having a greater dGb than QHs. There were no significant differences between breeds in HAUC, Ge, dGe or Tmin. There were not enough horses to assess Tmax, as only five horses had glucose values that returned to baseline before the end of sampling at 240 minutes.

Table 2.7 Median (95% CI) for minimal model parameters in the five breeds. Different letters in each column indicate significant breed differences at P < 0.05.
Table 2.8 Median (95% CI) for breed differences in parameters describing the glucose deflection below baseline values in the FSIGTT. Significant pair-wise differences between breeds are indicated with different letters.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Gmin</th>
<th>Arabian</th>
<th>Morgan</th>
<th>WP</th>
<th>TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morgans</td>
<td>117.8&lt;sup&gt;a&lt;/sup&gt; (90.1-199.7)</td>
<td>94.8&lt;sup&gt;b&lt;/sup&gt; (71.8-207.3)</td>
<td>1.286&lt;sup&gt;b&lt;/sup&gt; (0.57-2.2)</td>
<td>1.615&lt;sup&gt;ab&lt;/sup&gt; (1.2-1.8)</td>
<td></td>
</tr>
<tr>
<td>WP</td>
<td>256.9&lt;sup&gt;bcd&lt;/sup&gt; (94.3-442.5)</td>
<td>175.5&lt;sup&gt;ab&lt;/sup&gt; (93.7-259.4)</td>
<td>0.406&lt;sup&gt;b&lt;/sup&gt; (0.22-2.5)</td>
<td>1.659&lt;sup&gt;b&lt;/sup&gt; (1.3-1.8)</td>
<td></td>
</tr>
<tr>
<td>TB</td>
<td>188.7&lt;sup&gt;acd&lt;/sup&gt; (122-234.1)</td>
<td>419.2&lt;sup&gt;ab&lt;/sup&gt; (201.2-713.2)</td>
<td>2.654&lt;sup&gt;ab&lt;/sup&gt; (0.86-4.3)</td>
<td>1.387&lt;sup&gt;ab&lt;/sup&gt; (1.1-1.7)</td>
<td></td>
</tr>
</tbody>
</table>

Evaluating OST Insulin Thresholds for Insulin Dysregulation

ROC curve analyses per breed at various time points is shown in Table 2.9. ROC curves could not be calculated for OST insulin thresholds for QHs because no QHs were defined as IR based on minimal model analysis of the FSIGTT. Similarly, OST insulin thresholds were not calculated for TBs as only 1 TB was classified as IR by minimal model analysis of the FSIGTT.

These insulin thresholds were based on obtaining the best threshold for optimizing sensitivity and specificity after bootstrapping the data and evaluating the ROC curve 1000 times for that specific breed. The 60, 75, and 90 minute times are presented in Morgans, Arabians, and WP based on our previous experience with insulin being elevated in IR vs IS horses at these time points,[154] in addition to these being the most common time points to be sampled clinically. In
Arabians, the highest mean sensitivity (87.5%) and second highest specificity (93.333%) were achieved at the 90 min time point with a 42 μIU/ml insulin threshold. For WP, at the 90 minute time point, there were two thresholds for insulin that maximized either sensitivity or specificity. An insulin threshold of 26.3 μIU/ml maximized sensitivity (88.9%), while having a specificity of 78.6%.

Additional time points (30, 150, and 180 minutes) are presented with the Morgans as previous work had shown an inability to distinguish cases from controls in the OST on the basis of an insulin threshold during the 60-120 minute time points which proved satisfactory for the other four breeds.[154] At 150 minutes and an insulin threshold of 17.65 μIU/mL, the sensitivity of the OST was a median of 90.9%, with a specificity of 17.65%.

A glucose threshold for ID was not examined as previous work has indicated that there is not a significant difference in glucose concentrations between IR and IS horses.[154]

Table 2.9 Median and 95% (low and high) CI for the sensitivity (se) and specificity (sp) of different insulin thresholds (Insulin) at various timepoints during an OST in three breeds (Morgans, Arabians, and WPs).

<table>
<thead>
<tr>
<th>Times</th>
<th>Insulin</th>
<th>sp.low</th>
<th>sp.median</th>
<th>sp.high</th>
<th>se.low</th>
<th>se.median</th>
<th>se.high</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morgans</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>10.65</td>
<td>55.56</td>
<td>81.82</td>
<td>100</td>
<td>27.27</td>
<td>60</td>
<td>88.89</td>
</tr>
<tr>
<td>60</td>
<td>8.4</td>
<td>9.091</td>
<td>36.36</td>
<td>64.29</td>
<td>66.67</td>
<td>90.91</td>
<td>100</td>
</tr>
<tr>
<td>75</td>
<td>16</td>
<td>55.56</td>
<td>83.33</td>
<td>100</td>
<td>20</td>
<td>50</td>
<td>83.33</td>
</tr>
<tr>
<td>90</td>
<td>10.85</td>
<td>10.93</td>
<td>36.36</td>
<td>69.12</td>
<td>44.39</td>
<td>80</td>
<td>97.48</td>
</tr>
<tr>
<td>120</td>
<td>13.3</td>
<td>33.33</td>
<td>64.29</td>
<td>90.91</td>
<td>28.54</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>150</td>
<td>17.65</td>
<td>9.977</td>
<td>36.36</td>
<td>66.67</td>
<td>66.67</td>
<td>90.91</td>
<td>100</td>
</tr>
<tr>
<td>180</td>
<td>4.55</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>10</td>
<td>40</td>
<td>71.46</td>
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<tr>
<td><strong>Arabians</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>35.5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>33.33</td>
<td>72.73</td>
<td>100</td>
</tr>
<tr>
<td>75</td>
<td>28.65</td>
<td>55.56</td>
<td>80</td>
<td>100</td>
<td>55.56</td>
<td>87.5</td>
<td>100</td>
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<tr>
<td>90</td>
<td>42</td>
<td>76.47</td>
<td>93.33</td>
<td>100</td>
<td>50</td>
<td>87.5</td>
<td>100</td>
</tr>
</tbody>
</table>
OST Responses

The AUC insulin was significantly lower in QHs than any other breed (P< 0.05), except for Morgans, although the AUC for Morgans was not significantly different from Arabians, WPs or TBs (Figure 2.11). At 90 minutes, IR Arabians and IR WP had a statistically significant higher concentration of insulin than IS horses of the same breed. In Morgans, insulin concentrations at 90 minutes was not significantly different between IR and IS animals. There was only one IR TB on the basis of minimal model analysis of the FSIGTT, so there was not enough power to identify a significant difference between the groups (Figure 2.12). The AUC glucose was significantly lower in QHs than any other breed, and Arabians had significantly lower AUC glucose than Morgans or TBs (both P< 0.05) (Figure 2.13).

<table>
<thead>
<tr>
<th>WP</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>90</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>25</td>
<td>25</td>
<td>26.3</td>
<td>32.7</td>
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<td>100</td>
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<td>50</td>
<td>66.67</td>
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<td></td>
<td>100</td>
<td>100</td>
<td>78.57</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>45.45</td>
<td>44.44</td>
<td>62.5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>77.78</td>
<td>77.78</td>
<td>88.89</td>
<td>77.78</td>
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<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.9 (cont’d)
Figure 2.11 Mean insulin concentrations (µIU/mL) and area under the curve (AUC) insulin difference over time during an OST in five breeds of horses. Different letters indicate significant differences at P<0.05.

<table>
<thead>
<tr>
<th>Breed</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>QH</td>
<td>a</td>
</tr>
<tr>
<td>Arabian</td>
<td>b</td>
</tr>
<tr>
<td>WP</td>
<td>b</td>
</tr>
<tr>
<td>Morgans</td>
<td>ab</td>
</tr>
<tr>
<td>TB</td>
<td>b</td>
</tr>
</tbody>
</table>
Figure 2.12 Mean insulin concentrations (µIU/mL) at 90 minutes during an OST in five breeds of horses between insulin sensitive (IS) and insulin resistant (IR) horses. Significant at P < 0.05.
Figure 2.13 Mean glucose concentrations (mg/dL) and area under the curve (AUC) insulin differences over time during an OST in five breeds of horses. Different letters indicate significant differences at P<0.05.
**Evaluating Insulin and Glucose Curve Trajectories**

The effect of various predictors on the entirety of the insulin and glucose curve trajectories is indicated in Table 2.10 and Table 2.11. All variables were tested individually and were continuous. Insulin concentrations had to be log transformed in order to perform trajectory analyses due to the large number of low insulin concentrations readings during the OST. TG concentrations as well as breed significantly affected the glucose trajectory (all $P < 0.001$). HMW adiponectin, age, as well as breed all significantly affected the insulin trajectory (all $P < 0.001$).

Table 2.10 Effects of various predictors on the entire glucose trajectory.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Glucose Trajectory Wald Chi square, degrees of freedom, p-value, SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>2.79, 3, 0.42</td>
</tr>
<tr>
<td>Breed</td>
<td>130.40, 12, &lt;0.001</td>
</tr>
<tr>
<td>Sex</td>
<td>2.30, 3, 0.51</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>5.31, 3, 0.15</td>
</tr>
<tr>
<td>TG</td>
<td>5.24, 3, &lt;0.001</td>
</tr>
<tr>
<td>NEFA</td>
<td>4.97, 3, 0.17</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.37, 3, 0.95</td>
</tr>
</tbody>
</table>

Table 2.11 Effects of various predictors on the entire insulin trajectory.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Insulin Trajectory Wald Chi square, degrees of freedom, p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>10.79, 3, &lt;0.001</td>
</tr>
<tr>
<td>Breed</td>
<td>107.15, 12, &lt;0.001</td>
</tr>
<tr>
<td>Sex</td>
<td>0.44, 3, 0.93</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>7.58, 3, &lt;0.001</td>
</tr>
<tr>
<td>TG</td>
<td>2.81, 3, 0.42</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.84, 3, 0.84</td>
</tr>
<tr>
<td>Leptin</td>
<td>9.89, 3, 0.02</td>
</tr>
</tbody>
</table>
Figure 2.14 Breed differences in baseline concentrations of: A) triglycerides (mg/dl), B) NEFAs (mmol/L), C) leptin (ng/ml) and D) HMW adiponectin (ug/ml). *= significantly different at P < 0.05.
Evaluating Breed Differences and Correlations Between Adipokines, Triglycerides (TG), Non-esterified Fatty Acids (NEFA), and Minimal Model Parameters

Significant breed differences existed between different adipokine and biochemical analyses (Figure 2.14). TG was lower in QHs than in WPs. NEFA concentrations were higher in QHs, Arabians, and TBs when compared with Morgans (all P <0.05). Arabians had higher leptin levels than QHs. QHs and Morgans had higher HWM adiponectin concentrations than Arabians, WP had higher HWM adiponectin concentrations than TBs (all P <0.05).

Discussion

Breed differences were apparent in glucose and insulin responses in the FSIGTT. For the FSIGTT, QHs had the lowest baseline insulin, and the highest SI of all the breeds and had lower peak insulins than all breeds except Morgans. QH also had some of the lowest peak glucose concentrations and AIRg, along with a higher DI. The larger muscle mass in QH, possibly in part due to a SINE insertion which decreases myostatin gene expression (a negative regulator of muscle size),[89] could contribute to these findings. In Arabians, a typically IR breed, there was a higher AIRg than in Morgans but not higher than in WP. A higher AIRg has been implicated in causing laminitis due to high insulin concentrations being linked to causing laminitis in normal horses and ponies.[31,32]

Glucose excursion below baseline did occur in all breeds, although it was not as notable as in other work.[4] This difference may have been due to the inclusion in this cohort of more IR animals who tended to become hypoglycemic at later time points (213 vs. 180 minutes) than the IS horses previously reported.[4] The HAUC may have been significantly different between breeds, but as most animals did not return to baseline levels of glucose by the end of testing, this may have affected our ability to detect a difference.
Based on a previous study performed by our group, horses were classified for the OST as ID if insulin concentration determined from a single time point sample obtained at 60, 75, 90 or 120 minutes post oral administration of 0.25 ml/kg bw Karo light corn syrup equal to or greater than 22.8, 18.7, 30.2 or 26.3 µIU/mL, respectively.[154] In the present study, we have refined thresholds for insulin concentration further with regards to breed, garnering sensitivities that are higher than what has been achieved by static testing or by our previous general guidelines for insulin thresholds.[54,154] For Arabians, an insulin concentration of >35.5, 28.65, or 42 µIU/mL at 60, 75, or 90 minutes, respectively, is suggestive of ID. For WP, an insulin concentration of >25, 25, 26.3 or 32.7 µIU/mL at 60, 75, 90 or 90 minutes, respectively, is suggestive of ID. Based on our findings, it was difficult to find a clinically useful threshold for determining ID in Morgans, and the 150 minute time point with an insulin concentration of >17.65 µIU/mL appeared to provide the best recommendation from the data presented here. Previous reports in which the OST was performed in a small number of Morgans have shown a greater difference in insulin responses.[11] Morgans in this study came from two different farms, with different lineages; it is possible that there may be a familial component to the insulin and glucose responses noted in Morgans in the previous study.

Breed differences in AUC and AIRg determined from the OST were noted. QH had the lowest insulin AUC of all breeds other than Morgans and the lowest glucose AUC of all of the breeds. This is consistent with the lower AIRg and higher SI as calculated from the FSIGTT. Many insulin responses for the QH were in fact undetectable during the OST. Previous work performing OSTs have used QHs as the control population.[11] Because their insulin and glucose responses are significantly different (lower) than many other breeds, their use as a control population may be best suited to when they are compared to other QHs exclusively.
Trajectory analysis of the glucose and insulin curves demonstrated not only differences in the shape of the curve between the breeds, but also the effects that different EMS defining traits and common confounders (age, sex, breed) have on the shape of the curve. Previous work evaluating OST trajectories found that age, breed, NEFA concentrations and TGs had effects on the glucose trajectory.[5] In this cohort, TG as well as breed had effects on the glucose trajectory whereas age did not, possibly because animals in the current study were younger than those in the earlier study in which increasing age was important in the change of the trajectory shape. NEFAs are normally suppressed after a meal,[156] and therefore may have been at lower circulatory levels than in the previous OST study in which a lower dose of corn syrup was used.[5] The previous work noted an effect of age, breed, sex, and TG on the insulin curve trajectory.[5] In contrast, HMW adiponectin, leptin, in addition to age, and breed were shown to have effects on the insulin curve trajectory in our study. Insulin causes increased total adiponectin release from adipose tissue,[157] and could explain the effects on the insulin curve noted here. Leptin, most commonly associated with obesity, has also been associated with IR, and by extension ID.[3,37,38,46]

Breed differences were apparent in concentrations of adipokines, NEFAs and TGs. In this study, HMW adiponectin was higher in WPs which is similar to previous findings,[5] but in contrast to at least one report that correlated adiponectin more strongly with insulin sensitive individuals.[3] HMW adiponectin was also negatively correlated to increasing adipocyte size which is supportive of its traditionally being correlated to insulin sensitive individuals. Leptin concentrations were higher in the Arabians in this study as compared to the QHs and increasing leptin concentrations have been correlated to increasing adiposity in the past.[3,46] Increased baseline NEFA concentrations in some of the more traditionally insulin sensitive breeds (QHs and
TBs) were surprising, as NEFAs have been found to be higher in obese IR horses in the past.[10] The higher concentration of TGs in WP as compared to QHs in this study is more in line with previous research regarding elevated concentrations in hyperinsulinemic ponies.[34]

Limitations to this study included a smaller number of WP and TB for performing the OST. Larger numbers may have allowed insulin threshold predictions more specific to TBs to be made. Horses and ponies came from several different farms across the country, although farms did have similar management styles.

Overall, clear differences exist in the EMS defining traits, including insulin and glucose responses to dynamic testing, for the five breeds examined in this study. The OST results should be interpreted in the light of breed related differences in insulin responses. Understanding how breed differences contribute to susceptibility to EMS and laminitis warrants further investigation.

Footnotes

a MinMod Millennium V6.0, Cedars-Sinai, Los Angeles, CA, USA

b Eli Lilly, Indianapolis, ID, USA

c Karo Syrup, ACH Food Companies Inc., Summit, IL, USA

d 2300 STAT Plus Glucose & Lactate Analyzer, YSI Incorporated, Yellow Springs, OH, USA

e Siemens Coat-A-Count Insulin Radioimmunoassay, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA

f HMW Adiponectin, EMD Millipore, Billerica, MA, USA

g Serum triglycerides determination, Sigma-Aldrich, St. Louis, MO, USA

h Non-esterified fatty acids, Wako Diagnostics, Richmond, VA, USA

i Leptin RIA, EMD Millipore, Billerica, MA, USA
Acknowledgements:

The authors would like to thank Dr. Nichol Schultz for her assistance with the trajectory analyses.
3. Muscle and Adipose Histology and Relationship with Insulin Sensitivity and Breed

Evaluation of adipocyte and gluteal muscle histology differences in light of insulin sensitivity and total body fat composition in five breeds of horses

Summary

Background: Equine metabolic syndrome (EMS) and associated insulin dysregulation (ID) have been identified as the most common cause of laminitis and appear more prevalent in certain breeds. Muscle and adipose tissue have large roles in glucose and insulin regulation, and their histological characteristics have correlations to insulin dynamics in humans, but little is known as to whether these relationships are similar in the horse.

Objectives: To compare adipose tissue and gluteal muscle histology (cell or fiber area, enzyme activity, fiber type proportions) to body condition score (BSC), total body fat mass, and calculated values for assessment of insulin response from the frequently sampled intravenous glucose tolerance test (FSIGTT) in five breeds of horses.

Study Design: Analytic randomized prospective study

Methods: Eighty-two horses/ponies of a range of insulin sensitivities of five breeds had tail head adipose tissue biopsies, and twenty-eight horses had middle gluteal muscle biopsies. Deuterium dilution analysis (for total body fat percentage; TBFM), and an FSIGTT was performed in all
horses. Statistics included: Shapiro-Wilk normality testing, a Kruskal-Wallis test, Kolmogorov-Smirnov analyses, and one way ANOVAs, MANOVAs, and Spearman correlations. Significance was set at $P<0.05$.

**Results:** Overall BCS was weakly to moderately correlated to SI, AIRg, and mean adipocyte size ($\rho = -0.33$, $P=0.001$; $\rho = 0.48$, $P<0.001$; $\rho = 0.39$, $P<0.001$), while TBFM was not correlated to SI ($\rho = -0.14$, $P=0.21$), and weakly correlated to AIRg and adipocyte size ($\rho = 0.26$, $P=0.02$; $\rho = 0.29$, $P=0.008$). Breed differences existed in adipocyte area, with Quarter Horses having a significantly smaller mean adipocyte area than Arabians and Welsh Ponies but not Thoroughbreds or Morgans ($P<0.001$). Similarly, the distributions of adipocyte area only differed between QH and all other breeds ($P<0.001$). Adipocyte area was weakly related to SI ($\rho = -0.33$) and moderately to AIRg ($\rho = 0.48$) (all $P<0.001$) amongst all of the breeds. TBFM was poorly correlated to adipocyte area of all breeds combined ($\rho = 0.28$, $P=0.018$), but moderately correlated to QH adipocyte area ($\rho = 0.6$, $P=0.015$), not correlated to Arabian adipocyte area ($\rho = -0.004$, $P=0.98$), WP ($\rho = -0.033$, $P=0.94$), Morgans ($\rho = 0.433$, $P=0.072$), or TB ($\rho = 0.2$, $P=0.78$). Adipocyte area was significantly moderately correlated to Type 1 muscle fiber percent area ($\rho = 0.524$, $P = 0.004$). FSIGTT baseline insulin concentrations were moderately correlated to Type 1 muscle fiber percent area ($\rho = 0.4$, $P = 0.031$) and Type 1 muscle fiber proportion ($\rho = 0.45$, $P = 0.02$). There were no significant correlations to SI, AIRg, DI, or SG. No breed differences existed between muscle fiber type area or fiber type proportion, but within breed differences were present.

**Main Limitations:** One depot of adipose tissue and muscle tissue were sampled at a single time point.
Conclusions: Larger adipocyte size was not strongly correlated to SI as is noted in humans. TBFM and BCS may explain some of the variation of adipocyte size and SI but other factors also must also contribute to these differences. Muscle fiber type total percent area and proportion did not correlate to SI. QH did have a greater total percent area of type 2B to type 2A muscle fibers.

Introduction

Equine metabolic syndrome (EMS) and associated insulin dysregulation (ID) has been identified as the most common cause of laminitis.[47] In humans and horses, muscle and adipose tissue have large roles in glucose and insulin regulation, with skeletal muscle acting as the major site of insulin-stimulated glucose uptake in the postprandial state,[158] [159-161] and adipose acting as a glucose and lipid reservoir.[162,163] In humans, some studies have found skeletal muscle type 1 fibers have a greater ability to efficiently utilize glucose, but are similar to type 2 fibers with respect to sensitivity to insulin.[164] Other human studies have found muscle fiber types relate to differences in insulin sensitivity, particularly in certain races. African American women have lower insulin sensitivity and lower muscle fiber mitochondrial oxidative capacity, as well as having a greater proportion of Type 2 vs Type 1 muscle fibers.[79,165,166] This same lower sensitivity to insulin and fiber type proportions has also been seen in rats.[167] While skeletal muscle fiber type proportions within various muscles has been described for different breeds of horses,[80,168-174] studies comparing a breed specific insulin sensitivities to their muscle fiber type characteristics are lacking. Some of the only studies to date have examined normal vs. PSSM affected horses, noting that Belgians had greater insulin sensitivities [81] than previously reported in Quarter Horses [82] as assayed by a hyperinsulemic-euglycemic clamp. It was purported that this difference was due to a higher proportion of Type 2A (more oxidative) to
Type 2B muscle fibers in the Belgian horses, but no other breeds have been examined to assess the veracity of this relationship. In addition to differences in fiber type proportions, humans with metabolic syndrome have been shown to have larger muscle fibers (Types 1 and 2A) due to increased intramyocellular lipid deposition.\[175\] \[176\] To the authors’ knowledge, no information has been published in horses relating muscle fiber type/size to body condition score (BCS) or total body fat (TBFM).

In humans, obesity is related to hypertrophy of adipocytes, which in turn (undergo vascular dysfunction) outgrow their blood supply or lose the dilator effect on perivascular adipose vessels (enhancing peripheral resistance and blood pressure), become hypoxic, and release inflammatory mediators leading to insulin resistance.\[177\] In cats, obesity is also related to adipocyte hypertrophy and a pro-inflammatory gene expression profile.\[178\] In horses, although average adipocyte size in different adipose depots has been reported,\[96\] there is little published information about how adipocyte size may relate to BCS, TBFM, breed, adipokine, biochemical markers, and insulin sensitivity status. Therefore, the objectives of this study were: (1) to compare and contrast the histological characteristics of gluteal muscle and tail head adipose tissue (TAT) between breeds; (2) to determine if differences in gluteal muscle and TAT histological characteristics are correlated to measures of BCS, TBFM, and insulin sensitivity (via analysis of a frequently sampled insulin modified intravenous glucose tolerance test; FSIGTT); and (3) to determine if gluteal muscle and TAT characteristics possibly explain previously reported breed differences in measures of insulin sensitivity.
Materials and Methods

Overview of the study

Morphometrics and tissue biopsies were performed on day 1 of the study, with horses sedated with either intravenous (IV) xylazine\(^a\) (2.2 mg/kg bwt) or detomidine HCL\(^b\) (0.22 mg/kg) for the biopsies. Phenybutazone\(^c\) was given orally (2.2 mg/kg bwt) on the day of the biopsies and once a day for up to three days thereafter if needed for pain management. Oral sugar tests (OST) and frequently sample intravenous glucose and insulin tolerance tests (FSIGTT) were performed on days 2-5 in a randomized block design. Deuterium dilutions were performed on day 5 after dynamic glucose testing. Twenty-eight horses had all tests performed. Fifty-four horses had all tests performed except for the muscle biopsy. All horses had hay, but not water, removed from the stall at 10 PM the night before any dynamic testing. Signalment and medical history relevant to laminitis were recorded for each horse.

Morphometrics

Horses were assigned a BCS by a single investigator.\(^{[13]}\)

Tail-head Adipose Biopsies

A sample of subcutaneous adipose tissue was obtained from the adipose tissue depot located lateral and adjacent to the tail head using small rongeurs. A 2x2 inch of the overlying haircoat was clipped and the skin shaved on either side of the tail head. Samples (400-600 mg wet weight) were collected via rongeurs under aseptic conditions after desensitization of the area with a local skin block and making a small skin incision (1.0 cm). A single suture, placed in the skin after completion of the biopsy, was removed in 5-14 days. Adipose tissue was placed in
formalin for 24 hours, then 60% sucrose solution for 4 hours, and then washed in 60% ethanol for 5 washes and stored in 60% ethanol.

**Tail-head Adipose Tissue Characterization**

Adipose tissue was sectioned in 10 -12 um slices at -20 degrees Celsius using a cryostat (ThermoFisher Cryotome FSE) and stained with H&E. Images were obtained with a Nikon microscope using the SPOT 5.1 program. Area, and minimum and maximum diameter for one hundred cells was determined using Image J software,[179] and the mean for each horse and breed was calculated. Distribution of adipocyte sizes per breed was also examined.

**Gluteal Muscle Biopsy**

A sample of skeletal muscle tissue (500-800 mg) was obtained by percutaneous (Bergstrom) needle biopsy technique from the middle gluteal muscle at a uniform depth (compartment 2).[180,181] A 3x3 inch area of the overlying hair coat was clipped and the skin shaved. Samples (500-800 mg wet weight) were collected under aseptic conditions after a local skin block and a small skin incision (1 cm). The samples were collected at a uniform depth and site, and a single suture placed in the skin after completion of the biopsy and removed in 4-14 days. The muscle was either attached via Tissue-Tek Optimum Cutting Temperature (OCT) to a small round cork and frozen in isopentane cooled with liquid nitrogen, or submerged in a cryovial filled with OCT and frozen in a similar manner. Samples were stored at -80ºC.
Gluteal Muscle Characterization

Serial sections (7 μm) of muscle were cut in a -20 cryostat. Samples were incubated at pH 4.44 pH, and stained for myosin ATPase activity, with a post stain pour on/pour off of a 50% eosin solution to identify Type 1, 2A, and 2B muscle fibers. Slides were scanned using an automatic slide scanner. JPEGs were created for analysis of morphometrics by PAX-it!². A minimum of two hundred and fifty muscle fibers per horse were used to determine the total percent area and proportions of Type 1, 2A, and 2B fibers.

Frequently Sampled Insulin-Modified Intravenous Glucose Tolerance Test (FSIGTT)

To determine insulin sensitivity, our group used the FSIGTT procedure[9; 10] first described by Hoffman et al.[144] followed by Minimal Model Analysis (MinMod Millennium V6.0)⁶ to analyze insulin and glucose responses. A jugular catheter was placed prior to the start of the test. A baseline blood sample for insulin and glucose measurements was collected (0 minutes), followed by administration of a 300 mg/kg intravenous (IV) dextrose dose, with a 20 μIU/kg IV insulin⁷ dose given 20 minutes later. Blood samples (for insulin, and glucose measurements) were collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, 180, 210, and 240 minutes after dextrose had been administered.

Biochemical and Hormonal analysis

Blood was collected into tubes with no additives (insulin, NEFAs, adiponectin, leptin, TGs) or into Na heparin or NaF/K-oxalate (glucose) tubes. Blood was centrifuged on site and serum or plasma pipetted and stored at -80°C until analysis. Glucose concentrations were
determined via the glucose oxidase method (2300 STAT). Insulin concentrations were determined via a radioimmunoassay validated for the horse (Coat-A-Count RIA). [14-16]

Glucose and Insulin Dynamics

Minimal model analysis (MinMod Millennium V6.0 and WinSAAM; http://www.winsam.org) were used to analyze the glucose and insulin data derived from testing in each horse. These analyses yielded estimates of insulin sensitivity (SI (min⁻¹/mU/L); insulin-mediated glucose disposal) and the acute insulin response to glucose (AIRg ([mU/L]·min⁻¹); a measure of the degree of insulin secretory response to glucose). Horses were either classified as either insulin sensitive (IS; SI ≥ 1) or insulin resistant (IR; SI ≤ 0.99).

Additionally, glucose deflection below baseline was also examined during the FSIGTT. Parameters studied during this deflection included: Gb, baseline glucose, Gmin, the lowest glucose concentration below baseline, Tmin, the time point at Gmin, Ge, the glucose at 240 min (sampling endpoint), dGb, the percent deflection of glucose below Gb, calculated as (Gb-G min) x 100/Gb, dGe, the percent deflection of glucose below Ge, calculated as (Gmin- Ge) x 100/Ge, HAUC, area under the curve below baseline glucose and Tmax, the time point where glucose deflection returned to baseline. [4]

Deuterium Dilution

Deuterium dilution was performed as per Dugdale et al. Briefly, food and water was removed before the test and body condition score (BCS) and body weight were determined. A baseline blood sample for deuterium was obtained and deuterium administered (0.11-0.13 g/kg bwt IV, based on BCS) via a jugular catheter followed by 100 mls of sterile saline. Four hours later, a
post deuterium administration blood sample was obtained. Blood samples were centrifuged, pipetted, and frozen at -80ºC until analysis could be performed (Metabolic Solutions, Inc., Nashua, NH, USA). Total body fat mass (TBFM) was calculated as per Dugdale et al.[18] Total body water (TBW) was first calculated: TBW (moles) = (WA/18.02a) x ((δ_{dose} – δ_{tap})/ δ_{post} – δ_{pre}) where W is the amount of water (grams) used to dilute the dose, A is the amount of dose (grams; g) administered to the horse, a is the amount of dose (g) diluted for analysis, δ_{dose} is the delta D from the deuterium stock solution, δ_{tap} is the delta D from the tap water, δ_{post} is the delta D from the plasma after 4 hours of testing, and δ_{pre} is the delta D from the plasma before testing. TBW (moles) was converted to kilograms (kg): TBW (kg) = TBW (moles) x 18.02/1000 g/kg. To calculate a deuterium corrected TBW (kg) (TBW_D) we divided TBW (kg) by 1.04. Fat free body mass (FFBM) was calculated as: FFBM = TBW_D (kg)/0.732. Total body fat mass (TBFM) was determined as the difference between body mass (from the scale) and FFBM. Horses were removed from analyses involving TBFM when the deuterium determination was not performed (N=4) or in instances in which the calculation for TBFM resulted in negative or zero values (N=12).

Statistics

Shapiro-Wilk was performed for normality testing. A Kruskal-Wallis test and MANOVA were used to compare mean adipocyte and muscle fiber sizes, as well as proportions of muscle fiber types between breeds. A Kolmogorov-Smirnov analysis was performed to ascertain if there were statistical differences between breeds in regards to adipocyte area distributions. A one way ANOVA was used to examine breed differences in adipokines, TGs, and NEFAs. Spearman correlations were used to look for relationships between the histologic characteristics and measures.
from the minimal model analysis of the FSIGTT (SI, AIRg, DI, Sg), TBFM, and BCS. Statistics were performed with dedicated software. Significance was set at P<0.05.

Results

Comparing Adipocyte Area Differences between Breeds

Breed differences existed in adipocyte area, with QHs having a significantly smaller mean adipocyte area than both Arabians and Welsh Ponies but not Thoroughbreds or Morgans (P<0.001) (Figure 3.1). Additionally, when comparing the entire distribution shape of adipocyte areas among breeds, QH were significantly different than all other breeds (P<0.001).

Comparing Adipocyte Minimum and Maximum Diameter Differences between Breeds

Breed differences in adipocyte minimum and maximum diameters are presented in Table 3.1. QH had significantly smaller maximum adipocyte diameter than all other breeds (P<0.001). Arabians had larger maximum adipocyte diameter than Morgans or WP (both P<0.001), but smaller maximum diameter than TBs (P=0.03). Morgans and WPs had significantly a smaller maximum adipocyte diameter than TBs (P<0.001).

Table 3.1 Average tailhead adipocyte diameters (μm) (+/- standard deviation). Different superscript letters indicate significant difference between breeds in that row (P <0.05).

<table>
<thead>
<tr>
<th></th>
<th>QH</th>
<th>Arabian</th>
<th>Morgan</th>
<th>WP</th>
<th>TB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maximum</strong></td>
<td>0.063&lt;sup&gt;a&lt;/sup&gt; (0.024)</td>
<td>0.093&lt;sup&gt;b&lt;/sup&gt; (0.034)</td>
<td>0.087&lt;sup&gt;c&lt;/sup&gt; (0.032)</td>
<td>0.086&lt;sup&gt;c&lt;/sup&gt; (0.032)</td>
<td>0.096&lt;sup&gt;d&lt;/sup&gt; (0.027)</td>
</tr>
<tr>
<td><strong>Minimum</strong></td>
<td>0.060&lt;sup&gt;a&lt;/sup&gt; (0.027)</td>
<td>0.090&lt;sup&gt;b&lt;/sup&gt; (0.034)</td>
<td>0.078&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.082&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.080&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

QHs had a significantly smaller minimum adipocyte diameter than all other breeds (P<0.001). Arabians had a significantly larger minimum adipocyte diameter than all other breeds (P<0.001). Morgans had a significantly smaller minimum adipocyte diameter than WP (P=0.03).
Figure 3.1 Adipocyte area (μm^2) in 5 different breeds of horses (N=82). *significantly different at P<0.05.
Figure 3.2 Adipocyte area (µm^2) versus total body fat mass (TBFM) in all horses (N=90) (A), total body fat mass (TBFM) in Quarter Horses (QH) (B), SI (C), and AIRg (D).

Comparing Measures of Adiposity (BCS and Deuterium Dilution) to Each Other and Outcome Measures from the FSIGTT

The average BCS was 5.8/9. The average %TBFM was 18.4. BCS and TBFM calculated from the deuterium dilution technique were moderately correlated (rho=0.47, P<0.001). Overall BCS was weakly to moderately correlated to SI and AIRg, (rho = -0.33, P=0.001; rho = 0.48,
P<0.001), while TBFM was not correlated to SI (\( \rho = -0.14, P = 0.21 \)) and weakly correlated to AIRg (\( \rho = 0.26, P = 0.02 \)) (Figure 3.2). Arabians had significantly higher TBFM% than QHs (P = 0.001) and WPs (0.04). TBFM results were not available for sixteen horses that either did not have the test performed or had a negative TBFM and therefore were excluded from analysis.

**Comparing Measures of Adiposity to Adipocyte Area in Different Breeds**

TBFM was weakly correlated to mean adipocyte area for all breeds combined (\( \rho = 0.29, P = 0.018 \)), but moderately correlated to QH adipocyte area (\( \rho = 0.6, P = 0.015 \)) (Figure 3.2), and not correlated to Arabian adipocyte area (\( \rho = -0.004, P = 0.98 \)), WP (\( \rho = -0.033, P = 0.94 \)), Morgan (\( \rho = 0.433, P = 0.072 \)), or TB (\( \rho = 0.2, P = 0.78 \)).

**Comparing Adipocyte Area to Measures of Adiposity and Outcome Measures from the FSIGTT**

Mean adipocyte area for all breeds was weakly correlated to TBFM (\( \rho = 0.29, P = 0.008 \)), and weakly correlated to BCS (\( \rho = 0.39, P < 0.001 \)), SI (\( \rho = -0.39, P < 0.001 \)), and AIRg (\( \rho = 0.37, P < 0.001 \)) (Figure 3.2).

**Correlations between Adipocyte Size and Muscle Fiber Type**

Adipocyte area was significantly moderately correlated to Type 1 muscle fiber percent area (\( \rho = 0.524, P = 0.004 \)), but not to Type 2A or Type 2B muscle fiber percent area or proportions (Table 3.2).

**Comparing Gluteal Muscle Fiber Type Total Percent Area and Proportion Differences**

There were no significant differences for individual fiber type total areas between breeds (P = 0.17). When comparing muscle fiber type total percent area within breeds (Table 3.2), in QHs
there was a lower percent area of type I fibers as compared to Types 2A (P = 0.01) and 2B (P<0.001), and a lower Type 2A area than type 2B (P<0.001). Arabians were shown to have a lower percent area of Type 1 as compared to Type 2B (<0.001), and Type 2A percent area was lower than Type 2B (P=0.03) and in Morgans there was a lower Type 1 percent area than Type 2B (P = 0.002). Finally, there was a lower Type 1 percent area than Type 2A (P=0.001) or 2B (P<0.001) in WPs and a lower percent area Type 2A vs Type 2B fibers (P=0.01).

When comparing muscle fiber type proportions, there were no significant differences between breeds (P = 0.1) whereas when comparing muscle fiber type proportions within breeds (Table 4), there was a lower proportion of Type 1 fibers when compared to Type 2A (P=0.004) or 2B (P<0.001) in QHs. There were no significant differences in the proportion of Type 1, Type 2A and Type 2B fiber types in Arabians and Morgans whereas a lower proportion of Type 1 fibers vs. Type 2A (P=0.001) or Type 2B (P=0.004) was evident in WPs.

Comparing Gluteal Muscle Fiber Type Area and Proportions to Outcome Measures from the FSIGTT

Baseline insulin levels from the FSIGTT moderately correlated to Type 1 percent area (rho = 0.4, P = 0.031) and Type 1 proportion (rho = 0.45, P = 0.02). No significant correlations were seen to SI, AIRg, DI, SG, or any parameters related to glucose deflection below baseline, although there was a trend towards significance when examining the correlation between SI and Type 1 muscle fiber total percent area (rho = -0.35, P = 0.06).
Table 3.2 Average total percent area (± standard deviation) of Type 1, 2A, and 2B middle gluteal muscle fibers from biopsies in 28 horses of four breeds. Different superscript letters indicate significant differences between muscle fiber types within a column/breed (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>QH</th>
<th>Arabian</th>
<th>Morgan</th>
<th>WP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>4.9a</td>
<td>17.5a</td>
<td>22.2a</td>
<td>10.1a</td>
</tr>
<tr>
<td></td>
<td>(5.2)</td>
<td>(6.4)</td>
<td>(17.5)</td>
<td>(4.7)</td>
</tr>
<tr>
<td>Type 2A</td>
<td>25.8b</td>
<td>32.1ab</td>
<td>37.4ab</td>
<td>36.6b</td>
</tr>
<tr>
<td></td>
<td>(17.2)</td>
<td>(14.5)</td>
<td>(9.3)</td>
<td>(4.3)</td>
</tr>
<tr>
<td>Type 2B</td>
<td>65.4c</td>
<td>50.5c</td>
<td>47.7b</td>
<td>57.7c</td>
</tr>
<tr>
<td></td>
<td>(16.8)</td>
<td>(15.2)</td>
<td>(20.8)</td>
<td>(12.4)</td>
</tr>
</tbody>
</table>

Table 3.3 Average proportions (± standard deviation) of Type 1, 2A, and 2B gluteal muscle fibers from biopsies in 28 horses of four breeds. Different superscript letters indicate significant differences between muscle fiber types within a column/breed (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>QH</th>
<th>Arabian</th>
<th>Morgan</th>
<th>WP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>11.8a</td>
<td>27.4</td>
<td>27.8</td>
<td>16.5a</td>
</tr>
<tr>
<td></td>
<td>(9.8)</td>
<td>(8.7)</td>
<td>(18.4)</td>
<td>(6.7)</td>
</tr>
<tr>
<td>Type 2A</td>
<td>35.7b</td>
<td>31.8</td>
<td>33.5</td>
<td>42.8b</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(12.7)</td>
<td>(4.3)</td>
<td>(13.3)</td>
</tr>
<tr>
<td>Type 2B</td>
<td>52.5b</td>
<td>40.8</td>
<td>38.7</td>
<td>40.7ab</td>
</tr>
<tr>
<td></td>
<td>(15.9)</td>
<td>(14.8)</td>
<td>(21.2)</td>
<td>(14.1)</td>
</tr>
</tbody>
</table>

Discussion

Across all breeds examined in the current study, average adipocyte size was 83 μm and average adipocyte area was 5364 μm², with the largest adipocyte diameter being 144 μm. TAT area was reported to be 3537 +/- 1375 μm² previously[96]; whereas in the current study TAT
area was larger, potentially due to the breeds examined. When assessed by breed, average TAT area (μm^2) was 3600 in QH, 6755 in Arabians, 5379 in Morgans, 5605 in WPs, and 6281 in TBs. Differences between our findings and those of Bruynsteen et al. may be attributed to differences in the study cohort which, in the study of Bruynsteen et al consisted of 12 horses of mixed breeds (an assortment of warmbloods, a TB, a trotter, a Halflinger, Selle Francais, with half overweight or obese and half normal BCS) and of various ages (1-25 years of age), which had less horses that were from traditionally IR breeds and where the smaller number of animals could have an outlier become an influential point. The fact that adipocyte area was only weakly correlated to SI and AIRg suggests that the proposed mechanism in humans driving metabolic syndrome (adipocyte hypertrophy and subsequent release of inflammatory mediators) may not occur in horses, or at least not in this particular adipose tissue depot. The absence (or reduced numbers) of macrophages in the TAT biopsies (as has been investigated in horses previously) would support this conclusion.[96]

In this study, the average percent TBFM was 18%, similar to that reported previously in ponies (6.6-18.9%).[182] The percent TBFM was higher than that in TB as determined by cadaver dissection (average 5.1%) or the combined average of TB and ponies (7.41%) reported by Webb et al.[183] It is likely that the higher average percent TBFM noted in the present study reflects the significantly higher TBFM % in Arabians vs. QHs, and the traditionally lean racing TB body type in horses in the earlier papers.

Several ponies and horses had negative TBFM as calculated with the deuterium dilution technique. Possible causes for this include: lean body type, dehydration, respiratory or fecal loss, inaccurate scale weights, and/or inadequate time for the deuterium to equilibrate with body water.[17,184,185] For this last point, depending on the amount of digesta, equilibration can occur between 3-5 hours after injection [185] (we sampled at 4 hours), and the digesta can account for 8-
18% of TBW.\textsuperscript{184} Although food was withheld the night before and both food and water were withheld during the deuterium dilution test, horses may still have had a very variable amount of digesta present.

BCS has been shown to be a poor predictor of TBFM once the BCS was $> 6.83/9$, with 76% of horses with a BCS of less than that having good correlation to TBFM as determined via dissection.\textsuperscript{17} Although our average BCS was less than 6.83 in all of our breeds, we only saw a moderate correlation between BCS and TBFM. Furthermore, there were only weak to moderate correlations between measures of adiposity and SI. These findings support the idea that horses, like humans, can be metabolically healthy obese as well as thin and metabolically unfit, and that more than just obesity plays a role in metabolic health. The inclusion of generalized obesity as a mainstay of the current definition of EMS may be inappropriate in light of these findings, as animals that are thin but metabolically unfit may be missed.

Contrary to some previous reports, breed differences in fiber type area and proportions, were not apparent in this cohort. However, fiber type differences were noted within breeds in the current study. Additional muscle fiber samples, and statistically controlling for age may help discern breed differences. Snow \textit{et al.} (1980)\textsuperscript{80} demonstrated the highest percentages of fast twitch fibers (2A and 2B) to be present in QHS, followed by TBs, then Arabians, ponies, and hunter types. While the authors claimed there were differences between breeds, it is unclear what statistics were used to evaluate that claim. Similar proportions of fiber types were reported for ponies. Arabians in our study had a higher proportion of Type 2B (40) versus 2A (31) fibers, than did the Arabians in Snow \textit{et al} ’s study (37 and 47 respectively). This higher proportion of Type 2B fibers could be due to training, as our horses were young any training induced increase in the number of Type 2A fibers would have been minimal.\textsuperscript{80} Roughly equal proportions of Type 2A
to 2B fibers were reported in QH in the study by Snow et al.[80] whereas in the QH sampled in our study, Type 2B fiber proportions strongly trended towards being a higher proportion than Type 2A and comprised a significantly larger total percent area than Type 2A fibers. This is similar findings of Petersen et al.[90], in which Type 2B fibers were proportionally higher than Type 2A fibers in QHs. Our cohort of QHs also had a significantly lower proportion of Type 1 to either Type 2A or 2B fibers. In QH, a SNP and a SINE insertion in MSTN was associated with higher proportion of Type 2B and lower proportion of Type 1 muscle fibers.[89] Breed differences in fiber type proportion were not reported previously when horses without the SINE variation were examined (amongst Belgians, Thoroughbreds, and QH).[90] This finding is similar to our study with the exception that WP had a lower proportion of Type 1 to 2A fibers.

This study did not find correlations between muscle fiber type area or proportions and measures of insulin sensitivity or general adiposity. Although only one muscle was sampled (middle gluteal), as a large muscle used for locomotion with high energy needs, this seems the most appropriate muscle to see a possible relationship to insulin sensitivity if one existed. Although this finding agrees with one study in which fiber type proportions were not correlated to insulin sensitivity status in Belgians,[81] this is somewhat surprising, as QH are typically considered an insulin sensitive breed, and the SINE insertion has been linked with an increase in type 2B muscle fibers as mentioned above, as well as to higher adiponectin concentrations, and lower leptin, basal insulin, and insulin during an OST (NE Schultz and ME McCue unpublished data). Breeds that are known for larger muscling have also been reported to have glucose deflections below baseline during the FSIGTT procedure.[4] It was proposed that muscle fiber type measurements would correlate with some of the parameters related to these periods of
hypoglycemia, but this relationship was not evident in the present study, possibly because the periods of hypoglycemia were not as pronounced as in previous studies.[4]

TAT area was correlated to Type 1 muscle fiber total percent area in this study. Traditionally, Arabians have higher proportions of Type 1 muscle fibers in their gluteal area,[80] while Arabians are also a breed that has a high prevalence of IR and higher BCS. There was a trend for a negative correlation between Type 1 muscle fiber percent total area and SI (P = 0.06). Muscle samples in this study were obtained from a small number of horses, with a range of insulin sensitivities and BCS, factors which may have prohibited obtaining the power needed to detect a significant relationship between SI and fiber type measurements. Further evaluation of muscle fibers for infiltration of lipid droplets, which has been associated with metabolic syndrome in humans, would be needed to further evaluate the possibility of a relationship between fiber type, size, and insulin sensitivity status.[176]

Overall, measures of adiposity, adipocyte size (when examining this one subcutaneous depot), and middle gluteal muscle fiber type did not have strong correlations to tissue level insulin sensitivity and AIRg. Horses can demonstrate both a metabolically healthy obese, as well as a thin and metabolically unhealthy phenotype. Breed differences are apparent in adipocyte size, muscle fiber type proportion and muscle fiber type percent total area. Understanding of how these different breed differences contribute to the pathophysiology of EMS is worthy of further exploration.

**Footnotes**

aRompun, 100 mg/mL, Bayer, Shawnee Mission, KS, USA

bDormosedan, 10 mg/mL, Zoetis, Florham Park, NJ, USA
Phenylbutazone, 1 gram/tablet, Bimeda, Oakbrook Terrace, IL, USA

Nikon microscope, Melville, NY, USA

SPOT 5.1 program, Boulder, CO, USA

Tissue-Tek Optimum Cutting Temperature, Sakura Finetek, Torrence, CA, USA

Olympus slide scanner, Center Valley, PA, USA

PAX-it!2, Villa Park, IL, USA

Humulin R, U-100 vial, Eli Lilly, Indianapolis, ID, USA

YSI 2300 STAT, Life Sciences, Yellow Springs, OH, USA

Coat-A-Count RIA, Siemens, Washington, D.C., USA

Graph Pad Prism, La Jolla, CA, USA

R x 64 3.2.4, www.r-project.org

Acknowledgements

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4. EMS Related Muscle and Adipose

Differential Gene Expression

Gene Expression Differences and Functional Analysis of Adipose and Gluteal Muscle Tissues in Four Breeds of Horses with a Range of Insulin Sensitivities

Summary

Background: Equine metabolic syndrome (EMS) and associated insulin dysregulation (ID) has been identified as the most common cause of laminitis. Certain breeds appear to be susceptible to EMS, and we have identified breed differences in metabolic phenotypes. Muscle and adipose tissue have large roles in glucose and insulin regulation.

Objectives: Here we compare gene expression within the tailhead adipose tissue (TAT) and gluteal muscle in different breeds. TAT, and muscle biopsies were performed in 28 geldings from four breeds.

Study Design: Analytic randomized prospective study

Methods: Gene expression in gluteal muscle and TAT was measured using RNASeq. Differential gene expression was determined using HTSeq and Limma Voom. Functional analysis of genes was performed using Ingenuity Pathway Analysis (IPA).

Results: Each breed had uniquely differentially expressed genes in each tissue (7-1347 in adipose, 94-691 in muscle). In TAT, top differentially expressed networks in Arabians and Welsh Ponies (WP) were Carbohydrate Metabolism and Developmental Disorders/Lipid Metabolism respectively. Upstream analysis activation of cytochrome p450 reductase was evident in WP.
There was upstream activation of hypoxia-inducible factor 1-alpha and transforming growth factor beta 1 in Morgans and Arabians, with deactivation in Arabians and activation in Morgans of forkhead box protein 01, C-X-C Motif Chemokine Ligand 12, and growth hormone. In muscle, the top QH network was Lipid Metabolism, with upstream analysis showing deactivation of fenofibrate, pirinixic acid, and rosiglitazone. The top WP network was Energy Production/Lipid Metabolism.

**Main Limitations:** Only one adipose and skeletal muscle depot was investigated at one point in time.

**Conclusions:** Breed specific patterns of differentially expressed genes may contribute to ID.

**Introduction**

Equine metabolic syndrome (EMS) is characterized by a cluster of clinical signs including: obesity, regional adiposity, insulin resistance (IR), insulin dysregulation (ID), dyslipidemia, and a predisposition to laminitis.[1,6] Certain breeds appear predisposed to EMS (Morgans, Arabians, and Welsh Ponies [WP]), while other breeds appear to be less susceptible (Quarter Horses [QH] and Thoroughbreds [TB]).[6] While much work has been devoted to studying systemic responses of insulin and glucose to challenge tests for diagnosis of EMS/ID,[11,28-31,54,63,70,154] little has been done to examine the tissue level molecular pathophysiology of EMS, which may differ between breeds. Gluteal muscle is the primary site of insulin-stimulated glucose disposal and a key regulator of metabolic crosstalk with other body tissues (particularly adipose tissue) via secretion of myokines.[86,105,186] Adipose tissue’s importance in metabolic health and/or dysfunction is increasingly recognized with secretion of adipokines and inflammatory mediators driving metabolic syndrome in humans.[12,92,93,95,105,187-192] Currently, only a few equine studies
have examined isolated genes and/or proteins within the adipose and gluteal muscle tissues.[60,96,110-115] No studies to date have evaluated large amounts of the adipose or muscle transcriptome in different breeds of horses with varying susceptibilities to EMS in order to better understand different functional pathways that may explain breed predisposition to EMS/ID and laminitis.

What information we do have regarding equine gene expression differences in muscle and adipose does not always mirror the correlations between obesity, inflammation, and metabolic syndrome that have been described in humans. Researchers examining gene expression in equine skeletal muscle in EMS horses did not find any association between markers of inflammation or oxidative stress and obesity.[112,113] Researchers examining gene expression in the nuchal adipose tissue in EMS horses/ponies found increased levels of interleukin (IL)-6 but not of tumor necrosis factor alpha (TNF-alpha) when compared to non-EMS obese controls.[114] Other studies did not demonstrate differences between EMS and non-EMS horses and ponies in regards to gene expression, but did see depot differences in gene expression, with the nuchal adipose depot containing higher amounts of IL-1beta and IL-6.[110] While there are known genes that are associated with increased insulin sensitivity in muscle and brown adipose tissue (GLUT4, myostatin, irisin, some peroxisome proliferator-activated receptors), there are also those genes traditionally associated with increased insulin resistance (HIF1 alpha, TNF alpha, IL-1, IL-6).[93] Using RNA-seq to quantify gene expression, allowing for differential gene expression and pathway analysis across all genes in skeletal muscle and adipose tissue, offers the potential of moving beyond these commonly investigated genes to identify novel genes that may play important roles in the pathophysiology of EMS.[121-123] Therefore, the objectives of this study were to characterize and compare the transcriptome of skeletal muscle and adipose tissue in four
breeds of horses of different clinically noted susceptibilities to EMS. We hypothesized that there would be breed specific significant differences in differentially expressed genes present in muscle and adipose tissue that could be important at elucidating the pathophysiology of EMS.

**Materials and Methods**

**Horses**

Tail head adipose tissue (TAT) and middle gluteal muscle biopsies were collected from twenty-eight horses/ponies from 4 breeds (7 Quarter Horses [QH], 7 Arabians, 7 Morgans, and 7 Welsh Ponies [WP], all geldings). The ages (mean and range) of horses in this study were: QH (7, 2-20), Arabian (6.4, 2-21), Morgans (4, 2-7), and WP (7.4, 2-16).

**Experimental Design**

All horses were sampled between May and the first week of August at one of the following locations: Manhattan, KS (QH), East Lansing, MI (Arabians and Tb), Storrs, CT (Morgans), Chazy, NY (Morgans), and Olive Branch, MS (WP). Horses were not in work during the testing weeks and were maintained on their normal ration of predominantly grass hay (2-2.5% of body weight).

**Gluteal Muscle Biopsy**

A sample of skeletal muscle tissue (500-800 mg) was obtained by percutaneous (Bergstrom) needle biopsy technique from the middle gluteal muscle at a uniform depth (compartment 2).[180,181] A 3x3 inch area of the overlying hair coat was clipped and the skin shaved. Samples (500-800 mg wet weight) were collected under aseptic conditions after a local
skin block and a small skin incision (1 cm). The muscle was rinsed with saline and snap frozen in liquid nitrogen. The samples were collected at a uniform depth and site, and a single suture placed in the skin after completion of the biopsy. Sutures were removed in 4-14 days. Samples were stored at -80°C.

Tailhead Adipose Tissue Biopsies

A sample of subcutaneous adipose tissue was obtained from the adipose tissue depot located lateral and adjacent to the tail head using small rongeurs. A 2x2 inch of the overlying hair coat was clipped and the skin shaved on either side of the tail head. Samples (400-600 mg wet weight) were collected via rongeurs under aseptic conditions after desensitization of the area with a local skin block and making a small skin incision (1.0 cm). Adipose was immediately snap frozen in liquid nitrogen. A single suture was placed in the skin after completion of the biopsy. Sutures were removed in 5-14 days. Samples were stored at -80°C until analysis.

RNA Isolation and Quality Control

Samples (500 mg muscle, 600 mg adipose) were powdered in a liquid nitrogen pre-cooled stainless steel mortar. Total RNA was extracted using a modified Trizol method. Total RNA was then re-extracted using the Qiagen RNeasy mini kit and DNase treatment using the RNase-Free DNase Set per manufacturer’s instruction. Purity of total RNA was determined using a NanoDrop 2000 spectrophotometer (range = 1.96-2.1). A BioAnalyzer was used to assess RNA integrity using the relative integrity number (RIN). The mean muscle RIN was 8 (range 7.5-8.6), and the mean AT RIN was 7 (range 6.2-8.5).
**RNA Sequencing**

A RNASEq Library was prepared using 1 µg of total RNA per library. RNASEq analysis was performed on an Illumina HiSeq2500 platform, with 100bp, paired end reads, and three replicates of a sequencing depth of 20 million reads per each tissue.

**RNA Seq Data Analysis and Statistics**

The newest versions of software available in March of 2016 were used with the exception of Python, where version 2.7 was used. FASTQ files had adaptors removed and were trimmed with AdapterRemoval and were mapped to EquCab2.0 with BWA-MEM. Quality control of reads was performed with SAMtools flagstat. SAM files were then sorted and converted to BAM files. SAMtools was used to merge the read technical replicate files. A modified version of HTSeq that accepts both paired and unpaired reads (http://github.com/schae234/MixedHTSeq) was run to quantify read counts. Limma-voom and linear modeling identified uniquely differentially expressed genes based on breed. Ingenuity Pathway Analysis (IPA) with an FDR of 0.05 and a fold change of 1.5 was used for functional analysis.

**Results**

*Differences in Numbers of Breed Unique Differentially Expressed Genes in Tailhead Adipose Tissue and Gluteal Muscle*

Numbers of unique differentially expressed up- and downregulated genes by breed in both TAT and muscle are listed in Table 4.1 and Table 4.2. In TAT, downregulated genes represented 47.3% and upregulated genes represented 52.7% of all differentially expressed genes. In muscle,
downregulated genes represented 49.8% and upregulated genes represented 50.2% of all differentially expressed genes.

Table 4.1 Numbers of differentially expressed genes in the adipose tissue of a breed when compared to all other breeds (Arabians; Morgans; Quarter Horses (QH); and Welsh Ponies (WP)). Adjusted P values of < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Arabians-all</th>
<th>Morgans-all</th>
<th>QH-all</th>
<th>WP-all</th>
</tr>
</thead>
<tbody>
<tr>
<td>Downregulated</td>
<td>1173</td>
<td>311</td>
<td>7</td>
<td>235</td>
</tr>
<tr>
<td>Not differenti-</td>
<td>9674</td>
<td>11562</td>
<td>12185</td>
<td>11709</td>
</tr>
<tr>
<td>Upregulated</td>
<td>1347</td>
<td>321</td>
<td>2</td>
<td>250</td>
</tr>
</tbody>
</table>

Table 4.2 Numbers of differentially expressed genes in the gluteal muscle of a breed when compared to all other breeds (Arabians; Morgans; Quarter Horses (QH); and Welsh Ponies (WP)). Adjusted P values of < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Arabians-all</th>
<th>Morgans-all</th>
<th>QH-all</th>
<th>WP-all</th>
</tr>
</thead>
<tbody>
<tr>
<td>Downregulated</td>
<td>645</td>
<td>189</td>
<td>122</td>
<td>194</td>
</tr>
<tr>
<td>Not differenti-</td>
<td>8158</td>
<td>9107</td>
<td>9278</td>
<td>9125</td>
</tr>
<tr>
<td>Upregulated</td>
<td>691</td>
<td>198</td>
<td>94</td>
<td>175</td>
</tr>
</tbody>
</table>

*Top Canonical Pathways in Adipose Tissue and Gluteal Muscle*

Top canonical pathways in adipose tissue and gluteal muscle are represented in Table 4.3, Table 4.4, and Table 4.5. In TAT, only Morgan horses had a significant enough number of differentially expressed genes present in canonical pathways to analyze.
Table 4.3 Top significant canonical pathways in the adipose tissue of Morgans (significant with a Z score of ≥ 2 indicating activation).

<table>
<thead>
<tr>
<th>Canonical Pathway</th>
<th>Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Role of NFAT in Regulation of the Immune Response</td>
<td>2.236068</td>
</tr>
<tr>
<td>IL-8 Signaling</td>
<td>2.236068</td>
</tr>
<tr>
<td>B Cell Receptor Signaling</td>
<td>2.236068</td>
</tr>
<tr>
<td>p70S6K Signaling</td>
<td>2</td>
</tr>
<tr>
<td>VEGF Family Ligand-Receptor Interactions</td>
<td>2</td>
</tr>
<tr>
<td>PKCθ Signaling in T Lymphocytes</td>
<td>2</td>
</tr>
<tr>
<td>Colorectal Cancer Metastasis Signaling</td>
<td>2</td>
</tr>
<tr>
<td>Phospholipase C Signaling</td>
<td>2</td>
</tr>
<tr>
<td>Endothelin-1 Signaling</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4.4 Top differentially expressed genes in Morgan TAT involved in the canonical pathways (significant at FDR < 0.05)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exp Log Ratio</th>
<th>Exp p-value</th>
<th>Exp False Discovery Rate (FDR) (q-value)</th>
<th>Expected</th>
<th>Location</th>
<th>Type(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster of Differentiation (CD79A)</td>
<td>2.509</td>
<td>0.00191</td>
<td>0.0441</td>
<td>Up</td>
<td>Plasma Membrane</td>
<td>transmembrane receptor</td>
</tr>
<tr>
<td>Cluster of Differentiation (CD79B)</td>
<td>2.653</td>
<td>0.00174</td>
<td>0.0414</td>
<td>Up</td>
<td>Plasma Membrane</td>
<td>transmembrane receptor</td>
</tr>
<tr>
<td>Fos proto-oncogene (FOS)</td>
<td>2.755</td>
<td>0.00019</td>
<td>2</td>
<td>Up</td>
<td>Nucleus</td>
<td>transcription regulator</td>
</tr>
<tr>
<td>Major Histocompatibility Complex, Class II, DO Beta (HLA-DOB)</td>
<td>2.367</td>
<td>0.00013</td>
<td>9</td>
<td></td>
<td>Plasma Membrane</td>
<td>transmembrane receptor</td>
</tr>
<tr>
<td>Klotho Beta (KLB)</td>
<td>1.675</td>
<td>0.00106</td>
<td>0.0334</td>
<td>Up</td>
<td>Plasma Membrane</td>
<td>enzyme</td>
</tr>
<tr>
<td>Protein Kinase C Theta (PRKCO)</td>
<td>2.026</td>
<td>0.00169</td>
<td>0.0411</td>
<td>Up</td>
<td>Cytoplasm</td>
<td>kinase</td>
</tr>
</tbody>
</table>
Table 4.5 Top significant canonical pathways in the gluteal muscle of various breeds (significant with a Z score of ≥ 2).

<table>
<thead>
<tr>
<th>Canonical Pathway</th>
<th>Breed</th>
<th>Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoate Biosynthesis I</td>
<td>WP</td>
<td>3.029418</td>
</tr>
<tr>
<td>RAR Activation</td>
<td>WP</td>
<td>2.695744</td>
</tr>
<tr>
<td>Type I Diabetes Mellitus Signaling</td>
<td>WP</td>
<td>2.027686</td>
</tr>
<tr>
<td>Ubiquinol-10 Biosynthesis (Eukaryotic)</td>
<td>Morgans</td>
<td>3.396851</td>
</tr>
<tr>
<td>Circadian Rhythm Signaling</td>
<td>Morgans</td>
<td>2.644485</td>
</tr>
<tr>
<td>Glycine Degradation (Creatine Biosynthesis)</td>
<td>Morgans</td>
<td>2.368948</td>
</tr>
<tr>
<td>Sertoli Cell-Sertoli Cell Junction Signaling</td>
<td>Morgans</td>
<td>2.185582</td>
</tr>
<tr>
<td>Nur77 Signaling in T Lymphocytes</td>
<td>Morgans</td>
<td>2.178226</td>
</tr>
<tr>
<td>Hepatic Fibrosis / Hepatic Stellate Cell Activation</td>
<td>Morgans</td>
<td>2.152505</td>
</tr>
<tr>
<td>Melatonin Degradation II</td>
<td>Morgans</td>
<td>2.068827</td>
</tr>
<tr>
<td>Retinoate Biosynthesis I Arabians</td>
<td>Morgans</td>
<td>2.913187</td>
</tr>
<tr>
<td>Catecholamine Biosynthesis Arabians</td>
<td>Morgans</td>
<td>2.216487</td>
</tr>
<tr>
<td>Communication between Innate and Adaptive Immune Cells</td>
<td>Arabians</td>
<td>2.091928</td>
</tr>
<tr>
<td>OX40 Signaling Pathway</td>
<td>Arabians</td>
<td>2.091928</td>
</tr>
<tr>
<td>Serotonin and Melatonin Biosynthesis</td>
<td>Arabians</td>
<td>2.041036</td>
</tr>
<tr>
<td>Remodeling of Epithelial Adherens Junctions</td>
<td>QH</td>
<td>3.078021</td>
</tr>
<tr>
<td>Germ Cell-Sertoli Cell Junction Signaling</td>
<td>QH</td>
<td>2.904792</td>
</tr>
<tr>
<td>Granulocyte Adhesion and Diapedesis</td>
<td>QH</td>
<td>2.868217</td>
</tr>
<tr>
<td>Sertoli Cell-Sertoli Cell Junction Signaling</td>
<td>QH</td>
<td>2.859215</td>
</tr>
<tr>
<td>LPS/IL-1 Mediated Inhibition of RXR Function</td>
<td>QH</td>
<td>2.517784</td>
</tr>
<tr>
<td>LXR/RXR Activation</td>
<td>QH</td>
<td>2.362641</td>
</tr>
<tr>
<td>Glycine Degradation (Creatine Biosynthesis)</td>
<td>QH</td>
<td>2.26641</td>
</tr>
<tr>
<td>Epithelial Adherens Junction Signaling</td>
<td>QH</td>
<td>2.136748</td>
</tr>
</tbody>
</table>

**Top Upstream Regulators of Adipose Tissue and Gluteal Muscle**

Top upstream regulators in TAT and muscle are presented in Table 4.6 and Table 4.7. In adipose tissue, there was upstream analysis activation of cytochrome p450 reductase in WP and upstream activation of hypoxia-inducible factor 1-alpha (HIF1Alpha) and transforming growth factor beta 1 (TGFBI) in Morgans and Arabians, with inhibition in Arabians and activation in
Morgans of fork-head box protein 01 (FOX01), C-X-C Motif Chemokine Ligand 12 (CXCL12), and growth hormone. There was also had increased activation of TGFB2, TNF, WNT3a, and CTNNB1 and inhibition of VEGFA in Arabians and activation of MAPK in Morgans.

Table 4.6 Top upstream regulators that differ by breed in adipose tissue (significant if z score is ≤ -2-inhibited or ≥ 2-activated).

<table>
<thead>
<tr>
<th>Upstream regulators</th>
<th>WP Z score</th>
<th>Morgans Z score</th>
<th>QH Z score</th>
<th>Arabians Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>0.944097</td>
<td>2.605699</td>
<td>-1.95485</td>
<td></td>
</tr>
<tr>
<td>TGFβ1</td>
<td>0.738443</td>
<td>2.190766</td>
<td>2.139634</td>
<td></td>
</tr>
<tr>
<td>FOXO1</td>
<td>N/A</td>
<td>2.236068</td>
<td>-2.63363</td>
<td></td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>N/A</td>
<td>2.821941</td>
<td>-1.75689</td>
<td></td>
</tr>
<tr>
<td>HIF1A</td>
<td>N/A</td>
<td>2.252996</td>
<td>2.308506</td>
<td></td>
</tr>
<tr>
<td>CXCL12</td>
<td>N/A</td>
<td>2.387097</td>
<td>-2.1693</td>
<td></td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>1.073114</td>
<td>1.076906</td>
<td>2.308655</td>
<td></td>
</tr>
<tr>
<td>HMGA1</td>
<td>N/A</td>
<td>2.432701</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td>Growth hormone</td>
<td>N/A</td>
<td>2.195182</td>
<td>-2.23529</td>
<td></td>
</tr>
<tr>
<td>2-amino-5-phosphonovaleric acid</td>
<td>N/A</td>
<td>-2.20754</td>
<td>2.190365</td>
<td></td>
</tr>
<tr>
<td>Calcitriol</td>
<td>0.434057</td>
<td>1.594995</td>
<td>-2.27133</td>
<td></td>
</tr>
<tr>
<td>VEGFA</td>
<td>2.349666</td>
<td>2.360734</td>
<td>-1.10124</td>
<td></td>
</tr>
<tr>
<td>Tgf beta</td>
<td>2.026171</td>
<td>1.995381</td>
<td>2.308655</td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>0.519109</td>
<td>2.360734</td>
<td>-1.10124</td>
<td></td>
</tr>
<tr>
<td>Forskolin</td>
<td>0.561644</td>
<td>2.191893</td>
<td>-1.20696</td>
<td></td>
</tr>
<tr>
<td>kainic acid</td>
<td>2.572906</td>
<td>-1.33838</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>1.16518</td>
<td>2.3334</td>
<td>-0.31718</td>
<td></td>
</tr>
<tr>
<td>IL6</td>
<td>-0.86116</td>
<td>2.100219</td>
<td>0.844927</td>
<td></td>
</tr>
<tr>
<td>PGR</td>
<td>-1.2649</td>
<td>2.411765</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WNT3A</td>
<td>-0.35892</td>
<td>0.751439</td>
<td>2.55888</td>
<td></td>
</tr>
<tr>
<td>triamcinolone acetonide</td>
<td>-2.2188</td>
<td>-1.41421</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYC</td>
<td>2.325693</td>
<td>-1.26229</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creb</td>
<td>2.288943</td>
<td>-1.29459</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK1</td>
<td>2.152184</td>
<td>-1.41177</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pka</td>
<td>2.213211</td>
<td>-1.21976</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>2.178578</td>
<td>-1.25413</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>2.584574</td>
<td>-0.80202</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lysophosphatidic acid</td>
<td>2.346483</td>
<td>-1.03708</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-bromo-cAMP</td>
<td>2.30095</td>
<td>-1.0285</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRC</td>
<td>2.089956</td>
<td>-1.17627</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>2.255781</td>
<td>-0.98416</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY294002</td>
<td>-0.09759</td>
<td>-2.09179</td>
<td>-1.03226</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>IL1B</td>
<td>-0.29311</td>
<td>2.005927</td>
<td>0.860667</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>0.370079</td>
<td>0.660931</td>
<td>2.094441</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.9 (cont’d)

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>-2.17832</td>
<td>0.927173</td>
<td></td>
</tr>
<tr>
<td>SMARCA4</td>
<td>2.44949</td>
<td>0.624695</td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>2.210407</td>
<td>0.404041</td>
<td>0.445872</td>
</tr>
<tr>
<td>Cocaine</td>
<td>2.330669</td>
<td>-0.67555</td>
<td></td>
</tr>
<tr>
<td>Valsartan</td>
<td>-2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>2.193034</td>
<td>-0.79919</td>
<td></td>
</tr>
<tr>
<td>NFkB (complex)</td>
<td>0.867303</td>
<td>2.000563</td>
<td></td>
</tr>
<tr>
<td>Ca2+</td>
<td>2.762453</td>
<td>-0.10523</td>
<td></td>
</tr>
<tr>
<td>Isoprotenerol</td>
<td>2.186011</td>
<td>-0.66519</td>
<td></td>
</tr>
<tr>
<td>poly rI:rC-RNA</td>
<td>2.196089</td>
<td>0.636613</td>
<td></td>
</tr>
<tr>
<td>ERK1/2</td>
<td>0.131843</td>
<td>2.040358</td>
<td>-0.65221</td>
</tr>
<tr>
<td>25-hydroxycholesterol</td>
<td>2</td>
<td>-0.81098</td>
<td></td>
</tr>
<tr>
<td>nitric oxide</td>
<td>2.302484</td>
<td>-0.41421</td>
<td></td>
</tr>
<tr>
<td>CD40LG</td>
<td>2.410971</td>
<td>-0.28697</td>
<td></td>
</tr>
<tr>
<td>H89</td>
<td>-2.40925</td>
<td>0.285362</td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>2.019508</td>
<td>-0.66436</td>
<td></td>
</tr>
<tr>
<td>TGFB2</td>
<td>2.302484</td>
<td>0.641836</td>
<td></td>
</tr>
<tr>
<td>Bucladesine</td>
<td>2.218979</td>
<td>-0.35216</td>
<td></td>
</tr>
<tr>
<td>U0126</td>
<td>-2.44474</td>
<td>-0.10483</td>
<td></td>
</tr>
<tr>
<td>Anisomycin</td>
<td>2.190615</td>
<td>-0.27657</td>
<td></td>
</tr>
<tr>
<td>Dalfampridine</td>
<td>2.44949</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicuculline</td>
<td>2.395004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>platelet activating factor</td>
<td>2.374058</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mapk</td>
<td>2.185603</td>
<td>0.156174</td>
<td></td>
</tr>
<tr>
<td>MYD88</td>
<td>2.331633</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF BB</td>
<td>2.081823</td>
<td>-0.22384</td>
<td></td>
</tr>
<tr>
<td>Deferoxoxamine</td>
<td>2.176627</td>
<td>-0.02182</td>
<td></td>
</tr>
<tr>
<td>Arsenite</td>
<td>2.19449</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>2.170823</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phorbol esters</td>
<td>2.170763</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In muscle in QHs, upstream analysis showed inhibition of fenofibrate, pirinixic acid, and rosiglitazone.
Table 4.7 Top upstream regulators that differ by breed in gluteal muscle (significant if z score is ≤ -2 or ≥ 2).

<table>
<thead>
<tr>
<th>Upstream regulators</th>
<th>WP Z score</th>
<th>Morgans Z score</th>
<th>Arabians Z score</th>
<th>QH Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td>tretinoin</td>
<td>0.929303</td>
<td>-2.62821</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rosiglitazone</td>
<td>-2.42059</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fenofibrate</td>
<td>-2.40291</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pirinixic acid</td>
<td>0.439995</td>
<td></td>
<td>-2.40145</td>
<td></td>
</tr>
</tbody>
</table>

*Top Diseases and Biologic Functions in Adipose*

The top disease and biologic functions in TAT in each of the four breeds is in Figure 4.1 and Table 4.8. QH did not have any significant TAT differential gene expression that could be classified in the metabolic disease category.

Table 4.8 Top biological function pathways that differ by breed in adipose tissue (significant if z score is ≤ -2 or ≥ 2).

<table>
<thead>
<tr>
<th>Biologic Functions</th>
<th>WP Z score</th>
<th>Morgans Z score</th>
<th>QH Z score</th>
<th>Arabians Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>0.653009</td>
<td>-2.11519</td>
<td>0</td>
<td>-0.86122</td>
</tr>
<tr>
<td>cellular homeostasis</td>
<td>0</td>
<td>2.127648</td>
<td>0</td>
<td>-1.29794</td>
</tr>
<tr>
<td>differentiation of osteoclasts</td>
<td>0</td>
<td>1.1094</td>
<td>0</td>
<td>-2.18282</td>
</tr>
<tr>
<td>cell viability</td>
<td>0</td>
<td>2.860008</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cell movement of tumor cells</td>
<td>0</td>
<td>-0.44023</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>hyperplasia of tissue</td>
<td>0</td>
<td>2.432701</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>apoptosis of lymphocytes</td>
<td>0</td>
<td>-2.40483</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>urination disorder</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-2.4004</td>
</tr>
<tr>
<td>growth of lymphoid tissue</td>
<td>0</td>
<td>2.389564</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypertrophy</td>
<td>0</td>
<td>-2.38822</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hypertrophy of cells</td>
<td>0</td>
<td>-2.21321</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hypertrophy of tissue</td>
<td>0</td>
<td>-2.21321</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>growth of lymphoid organ</td>
<td>0</td>
<td>2.182821</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>homing of mononuclear leukocytes</td>
<td>0</td>
<td>-2.14494</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cell viability of cancer cells</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>binding of fibroblast cell lines</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>necrosis of pancreas</td>
<td>0</td>
<td>-2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 4.1 Top diseases represented by the differential gene expression patterns unique to each of the four breeds (dark blue=WP, light blue=Morgans, turquoise=QH, black=Arabians).
In TAT, top networks in Arabians and Welsh Ponies (WP) were Carbohydrate Metabolism and Developmental Disorders/Lipid Metabolism respectively (Table 4.9 and Figure 4.2).

Table 4.9 Top networks represented by the differential gene expression patterns unique to each of the four breeds in adipose tissue.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Score</th>
<th>Focus Molecules</th>
<th>Top Disease and Functions</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>WP</td>
<td>42</td>
<td>18</td>
<td>Developmental Disorder, Lipid Metabolism, Molecular Transport</td>
<td>Alp, Ap1, AXIN2, Cg, CRABP1, cytochrome C, EPS8L2, ERK, ERK1/2, FGF9, FSH, Growth hormone, ID1, IGFBP3, LDL, LDLR, MAFF, Map k, N-cor, Nr1h, Pdgf (complex), PDGF BB, PDK4, PLA2, PLA2G7, PLA2G16, PNPLA3, PPARGC1B, Proinsulin, SCD, SFRP4, SIRPB1, SRY2, THBS1, Vegf</td>
</tr>
<tr>
<td>Arabians</td>
<td>36</td>
<td>19</td>
<td>Carbohydrate Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism</td>
<td>ACAN, ADAMTS8, Akt, AMPK, C/cbp, CAMKK1, Collagen type I, Collagen type ix, CRABP1, Cyclin A, Cyclin D, EDIL3, FABP9, FMD, Growth hormone, IGFBP2, Integrin alpha V beta 3, ITGA11, JNK1/2, LEP, LIPE, NOV, NR1D1, Nr1h, PC, PCK1, PDK4, PEPCK, PFKFB1, PRKAA, Rxr, STAT5a/b, TGFBI, thyroid hormone receptor, TTPA</td>
</tr>
<tr>
<td>Morgans</td>
<td>22</td>
<td>11</td>
<td>Cell Morphology, Immunological Disease, Lymphoid Tissue Structure and Development</td>
<td>Adaptor protein 1, ADRA1, BCR (complex), CD79A, CD79B, Cyclin A, CYR61, ERK1/2, Fcer1, FCRL5, Gm-csf, Hspg, IgE, IgG, IgGl, IgG3, IGH, Igm, Immunglobulin, KLB, MAP2K1/2, Nfat (family), PI3K (family), PI5KB1, PLC gamma, PRKCQ, Rac, Rap1, RASGRP1, Sapk, Sos, SPDEF, SYK, ZAP, TRAT1, TSH</td>
</tr>
<tr>
<td>QH</td>
<td>15</td>
<td>5</td>
<td>Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance</td>
<td>AChR, amino acids, AMPK, ASNS, CEBPG, EIF2AK4, ELAVL1, ETS2, GNE, histidinol, L-arginine, L-histidine, leucine, linoleic acid, miR-22-3p (miRNAs w/seed AGCUGC), MTHFD2, MXI1, NAD+, NFKBIL1, PEX19, PFK M, PLA2, PLA2G16, PRKAG2, PROSC, PSMD9, RBM7, REV1, SEH1L, SESN2, SL C7A1, TFAP2A, TNF, TP53, ULK1</td>
</tr>
</tbody>
</table>
Figure 4.2 Connectivity of differentially expressed genes in the top network (Developmental Disorders/Lipid Metabolism) in adipose tissues in Welsh Ponies (N=7). Geometric figures in red represent upregulated and green indicate downregulated genes. Solid lines indicate direct connections and dashed lines indirect connections between the genes and their functions. Genes that aren’t colored were not identified in this data as being differentially expressed but were placed in the network based on evidence present in the IPA knowledge bank.

Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and/or beta (PPARCG1A and/or B) had significant differential expression in TAT or muscle in several breeds. 

PPARCG1A and B had increased expression in Arabian muscle (log fold change= 0.49, 0.69; adj p values= 0.02, 0.02). PPARCG1B had decreased expression in WP muscle (log fold change= -0.9, adj. p value= 0.03) and WP TAT (log fold change= -1.65, adj. p value= 0.008). A related gene,
Peroxisome proliferator-activated receptor gamma, coactivator 1 and estrogen-related receptor-induced regulator in muscle 1 (PERM1), had increased expression in Arabian muscle (log fold change= 0.4; adj. p value= 0.005), but decreased expression in WP muscle (log fold change= -0.41, adj. p value= 0.04).

In muscle, the top QH network was ‘lipid metabolism’. The top WP network was ‘energy production/lipid metabolism’ (Table 4.10).

Table 4.10 Top networks represented by the differential gene expression patterns unique to each of the four breeds in gluteal muscle.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Score</th>
<th>Focus Molecules</th>
<th>Top Diseases and Functions</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>WP</td>
<td>35</td>
<td>14</td>
<td>Energy Production, Lipid Metabolism, Small Molecule Biochemistry</td>
<td>11-dehydrocorticosterone,AICAR,AKR1C3.Akt,Alp,AMPK,androsterone,AQP4.Cg,CHRDL2,CNTFR,CPT1A,CPT1C,ERK1/2,FKBP5,FSH,G0S2,Growth hormone,HLA-A,IGFBP3,Immunoglobulin,Insulin,Lh,LRAT,malonyl-coenzyme A,MN1,MRC1,Nfkb (complex),P38 MAPK,PKD4,PFKFB,PFKFB3,SESN1,TNFRSF11B,Vegf</td>
</tr>
<tr>
<td>Arabians</td>
<td>34</td>
<td>14</td>
<td>Antimicrobial Response, Cell-To-Cell Signaling and Interaction, Cell-mediated Immune Response</td>
<td>5-hydroxytryptophan,AKR1C3,Akt,AQP4,ARNTL,CD4,CD3-TCR,Cg,CNTFR,Ctf2,DBP,DDC,EMCN,GRB14,HLA-A,HLA-DQA2,Immunoglobulin,KDM5D,KIF15,KIR3DL2,LIME1,Mapk,P38 MAPK,PFKFB3,Pkc(s),Selectin,SELL,Sh2b3,SHISA2,TCR, TMPRSS2,Trav3-3,Trv31,UGT2B15,Vegf</td>
</tr>
<tr>
<td>Morgans</td>
<td>43</td>
<td>18</td>
<td>Connective Tissue Development and Function, Skeletal and Muscular System Development and Function, Tissue Development</td>
<td>ABCA5,AKR1C1/AKR1C2,ANKRD2,ARNTL,Calcineurin protein(s),Creb,ERK1/2,FHL2,FRZB,FSH,GAB2,Insulin,Integrin,LEPR,Lh,MAP3K2,Mapk,MF2,Mek,MYH1,MYOZ2,NFAT (complex),NR4A1,Pde,PDE7B,PDGF BB,PER2,Pka,PLC,PLN,Proinsulin,TNFRSF11B,TPD52L1,TYRP1,Vegf</td>
</tr>
<tr>
<td>QH</td>
<td>41</td>
<td>18</td>
<td>Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry</td>
<td>ABCA5,ADRB,AKR1C1/AKR1C2,AMPK,ANGPTL4,ARH GAP32,CD36,CPT1A,ERK1/2,FRZB,GAB2,GPAM,Growth hormone,HDL-cholesterol,IgG1,IL1,IL13,Insulin,KLF11,LEPR,NFAT (complex),NR4A1,p85 (pik3r),PDGF BB,PKD4,PLC gamma,PRKG1,Pro-inflammatory Cytokine,Proinsulin,RHOU,Rxr,SDC4,SRC</td>
</tr>
</tbody>
</table>
Discussion

Breed unique significant gene differential expression was present in both tissues. To determine if these differences in gene expression are in part responsible for differences in measures of ID, or histopathology, direct correlations between genes and pathways based on expression measures and these variables will be examined in a larger cohort totaling 90 animals.

When examining the number of unique differentially expressed genes that were either up or down regulated as compared to all other breeds, QH had the lowest number. QH have been recognized as an insulin sensitive breed and have been used as the control group in some EMS papers.[5,11,193] This lower number of significantly differentially expressed genes suggests that traditionally insulin resistant breeds are made so by the additional breed specific up or down differentially expressed genes. This is further supported by findings in this study, as QH specific uniquely expressed genes do not appear to fall into the roles of being traditionally insulin sensitizing.

In the canonical pathway analyses, Morgans were the only breed to have a significant number of genes involved within TAT, while several breeds had upregulated pathways in muscle. In TAT, those top pathways and genes were associated with inflammation, specifically B and T cell receptors and Major Histocompatibility Complex II. In humans, systemic inflammation originating from specific adipose tissue depots is considered a component of metabolic syndrome.[43,93,95,188,189] In the horse, some studies have found certain adipose depots (nuchal) to have higher concentrations of inflammatory markers than others,[110] and this study’s findings lend support to an upregulation of inflammatory genes occurring in the TAT of Morgans.
Activation or inhibition of upstream regulators is predicted based on the differential expression of the genes in the data set when compared to known biological pathways. In TAT of WPs, cytochrome p450 was predicted to be activated. As a traditionally insulin resistant breed, this is somewhat surprising as in humans, metabolic syndrome has been associated with decreased mRNA levels of systemic cytochrome p450.[194] In Arabians and Morgans, both traditionally insulin resistant breeds, the prediction of activated HIF1alpha was more expected as increased HIF1alpha expression can induce interleukin 1beta expression which is linked to insulin resistance in mice.[195] Elevations of HIF1alpha in addition to being associated with insulin resistance, also suppressed adiponectin levels in other mouse studies, although that was not seen in this cohort.[196] Inhibition of HIF1alpha in mice ameliorated adipose dysfunction and resulted in weight loss.[197]

FOX01, a gene associated with increasing the rate of hepatic glucose production[198] and decreasing adipogenesis by inhibiting downstream effects of PPAR gamma,[198-200] was activated in Morgans and inhibited in Arabians. This finding is likely associated with the PPAR findings in muscle and adipose. While FOX01 has not been examined in horses, some studies have reported equine information on chemokines and growth hormone. Concentrations of chemotactic cytokine (CCL5) differed by depot in horses, with higher levels present in abdominal tissue.[96] In this study, CXCL12 was activated in the TAT of Morgans but not Arabians, supporting the possibility of an inflammatory profile in that adipose depot in Morgans, which agrees with the canonical pathway data. Growth hormone was also activated in Morgans, and supplemental growth hormone in horses has been shown to induce hyperinsulinemia.[201]

In muscle, there was predicted upstream inhibition of fenofibrate, pirinixic acid, and rosiglitazone in QH. These molecules are typically associated with increasing insulin sensitivity,
improving adipocyte function,[202] inhibiting PPARs,[203] and/or maintaining ideal mitochondrial function.[204] It is unclear why this inhibition would occur in a typically insulin sensitive breed, but perhaps these inhibitory changes are counter balancing the insulin sensitivity that is caused by other factors.

In TAT, top diseases represented included endocrine disorders, metabolic diseases (except in QHs), and inflammation. Horses in this cohort expressed a range of insulin sensitivities, so it is interesting to note that these areas were apparent in even a small sample size.

Top networks were generated from the data set generated from our study in a “bottom up” approach. They included: carbohydrate metabolism in Arabian TAT, disorders in lipid metabolism in WP TAT, lipid metabolism in QH, and energy production/lipid metabolism in WP. There was upregulation of PPARGC1a and B in Arabian muscle and decreases in expression of PPARGC1B in WP muscle and adipose. PPARGC1A is present in slow-twitch muscle fibers, of which Arabians traditionally have higher proportions.[80,205,206] Increased expression of PPARGC1B is associated with Type IIx muscle fiber formation[207] Knock-out mice models for PPARGC1B have shown increased serum triglyceride concentrations, which has been noted in this group of WPs previously.[193] In brown fat, PPARGC1B is associated with brown adipocyte differentiation, making its downregulation in WP, a typically insulin resistant breed, understandable.

One limitations of this study was the single sample of muscle and of adipose tissue depot obtained at a single time point that formed the basis of our results. The single site for adipose tissue sampling may be the most problematic as other equine papers have suggested that the nuchal adipose depot has interesting proinflammatory gene expression,[110] while human literature also suggests that omental or abdominal subcutaneous adipose depots may be the most important in the
development of insulin resistance.[95,189,208] The TAT depot was sampled here as preliminary data in our lab showed this depot to be as active as the nuchal region in regards to gene expression, with the added benefit of the biopsy site healing in a more cosmetic manner (which was desirable for client owned horses). Surprisingly, between breeds no differences were noted in gene expression related to genes that have been previously reported in the equine literature associated with insulin sensitivity/resistance, or insulin and glucose dynamics (glucose transporters, myostatin, irisin, interleukins, tumor necrosis factor alpha).[5,90,110,114,115] This may have been because we examined only a small number of horses in each breed for muscle and adipose gene expression analysis, and breed cohorts represented a range of insulin sensitivities, making it more difficult to detect significant differences in gene expression. It is possible more gene expression differences would have been evident if horses were analyzed based on insulin sensitivity status alone or in addition to breed. With the adipose tissue, some samples had poor RIN numbers. With RNA Seq, previous work has demonstrated that poor RINS (down to 6.4) allows all of the genes that could be significantly differently expressed to be noted but reduces the power to detect significance.[209]

Overall, breed differences were evident in the muscle and adipose transcriptome which highlighted novel genes and networks of significant interest to inform future work examining the molecular pathophysiology of EMS. With additional RNA Seq analysis of muscle and adipose planned in each of these four breeds, the power to detect correlations between gene expression and EMS defining traits, such as insulin and glucose responses during dynamic testing, minimal model parameters such as the acute insulin response to glucose and tissue level insulin sensitivity, as well as markers of lipid metabolism and adipokines, may become apparent, and could further guide future investigations.
Footnotes

a Qiagen RNeasy mini kit, Valencia, CA, USA

b RNase-Free DNase Set, Valencia, CA, USA

c NanoDrop 2000, Thermo Scientific, Waltham, MA, USA

d BioAnalyzer, Agilent Technologies, Wilmington, DE, USA

e Illumina HiSeq2500, San Diego, CA, USA

f Ingenuity Pathway Analysis, Quiagen, Redwood City, CA, USA
5. Conclusions and Future Work

Chapter Specific Conclusions

Breed Differences in Dynamic Testing for Equine Metabolic Syndrome (EMS) and Insulin Dysregulation (ID)

*Evaluation of an Arginine Stimulation Test for Assessment of Acute Insulin Response in Adult Horses*

Arginine HCL administered intravenously (IV) to adult horses was able to induce a significant rise in insulin concentration from baseline that was sustained for at least 15 minutes after administration. There was no significant difference in the insulin response to arginine between a 70 mg/kg or a 100 mg/kg bwt IV dose. The acute insulin response (AIR) to arginine was repeatable. Strong associations existed between the AIR arginine and the AIR for glucose (AIRglu) but not between the AIR arginine and insulin sensitivity (SI) as determined by an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT). These data suggest that the arginine stimulation test elicits a significant insulin response in adult horses, may provide an alternative method for assessment of the acute insulinenic response to glucose, but is not a good measure of tissue insulin sensitivity.

*Evaluation of a Modified Oral Sugar Test for Dynamic Assessment of Insulin Response and Sensitivity in Horses*

A modified oral sugar test (OST), with Karo syrup administered orally at 0.25 ml/kg and a single time point blood sample obtained at 60, 75, 90, or 120 minutes and which resulted in
Evaluation of Equine Breed Specific Insulin and Glucose Dynamics in Response to a Frequently Sampled Intravenous Glucose Tolerance Test and a Modified Oral Sugar Test

Breed differences existed in baseline adipokine concentrations and measures of lipid metabolism as well as responses to an FISGTT and OST. Typically, SI was higher and AIRglu was lower in QH when compared to other breeds. AIRglu was significantly higher in Arabians than Morgans, while Morgans had a significantly lower AIRglu when compared to WPs. The lowest glucose concentration attained in the FSIGTT (Gmin) was significantly different by breeds, with Arabians, Morgans, and WP having lower Gmins than Thoroughbreds (TB). Deflection of glucose below baseline (dGb) was significantly different by breed with Morgans having a greater dGb than QHs. For the OST, different insulin thresholds for ID were needed for each breed to maximize the sensitivity and specificity of the test. In Arabians, the highest mean insulin concentrations of \( \geq 22.8, 18.7, 30.2 \) or \( 26.3 \) µIU/mL, respectively, was indicative of ID in WP and Arabians but not Morgans. Glucose is not a useful measurement during the OST for evaluation of ID. Moderate correlations to insulin sensitivity derived from minimal model analysis of the FSIGTT and strong correlations to AIRglu were evident for area under the curve for insulin (AUCi), peak, and overall mean insulin. Weak correlations existed between glucose concentrations from the OST and SI and/or AIRglu. All indices had no better than moderate correlation to SI but had moderate to strong correlations to AIRglu. The OST appears to have better sensitivity to identifying IR horses than a baseline fasted blood sample of insulin, likely reflects breed differences in the elicited insulin response, is a good test for assessment of the insulin response but is not strongly associated with insulin sensitivity as calculated from the FSIGTT minimal model analysis.
sensitivity (87.5%) and second highest specificity (93.3%) were achieved at the 90 min time point with a 42 µIU/ml insulin threshold. For WP, an insulin threshold of 26.3 µIU/ml maximized sensitivity (88.9%), while having a specificity of 78.6% at 90 minutes. In Morgans, at 150 minutes and an insulin threshold of 17.65 µIU/mL, the sensitivity of the OST was a median of 90.9%, with a specificity of 17.65%. OST glucose and insulin trajectories and area under the curve also differed significantly by breed (lowest in QH) and were also affected by triglycerides (glucose), and age, breed, and HMW adiponectin concentrations (insulin). From these findings, it can be concluded that results from the OST, FSIGTT, and markers of lipid metabolism/adipokines should be interpreted in the light of breed related differences in insulin responses.

**Evaluation of adipocyte and gluteal muscle histology differences in light of insulin sensitivity and total body fat composition in five breeds of horses**

Overall, measures of adiposity, adipocyte size, and skeletal muscle fiber type did not have strong correlations to tissue level insulin sensitivity and AIRg unlike what is reported in humans. Breed differences existed in adipocyte area, with QHs having a significantly smaller mean adipocyte area than both Arabians and WPs but not TBs or Morgans. Similarly, the distributions of adipocyte area only differed between QH and all other breeds. Muscle fiber type total percent area and proportion did not correlate to SI. QH did have a greater area of type 2B to 2A muscle fibers. Total area and proportions of fiber types did not significantly differ between breeds. There were breed differences in adipocyte, but not muscle histology. The weak correlations between BCS, TBFM and SI and AIRg suggest that adiposity may not be a key factor in determining metabolic fitness in horses.
Gene Expression Differences and Functional Analysis of Adipose and Gluteal Muscle Tissues in Four Breeds of Horses

Each breed had uniquely differentially expressed genes in each tissue (7-1347 in adipose, 94-691 in muscle). In TAT, top networks in Arabians and Welsh Ponies (WP) were Carbohydrate Metabolism and Developmental Disorders/Lipid Metabolism respectively. Upstream analysis activation of cytochrome p450 reductase was evident in WP. There was upstream activation of hypoxia-inducible factor 1-alpha and transforming growth factor beta 1 in Morgans and Arabians, with deactivation in Arabians and activation in Morgans of forkhead box protein 01, C-X-C Motif Chemokine Ligand 12, and growth hormone. In muscle, the top QH network was Lipid Metabolism, with upstream analysis showing deactivation of fenofibrate, pirinixic acid, and rosiglitazone. The top WP network was Energy Production/Lipid Metabolism. Arabians had upregulation and WP down regulation of peroxisome proliferator activated receptor, coactivation receptor 1 (PPARGC1). Novel genes and pathways were determined and breed specific patterns of differentially expressed genes may contribute to ID.

Overall Conclusions

Testing for EMS/ID is an imperfect science, one where a clear gold standard has not been established and may never be achieved. In fact, intravenous challenge testing and the use of SI may be less appropriate than an oral challenge and assessment of AIRg for identifying “at risk” individuals. However, even with oral challenge testing, the findings presented here have provided evidence that a “one size fit all” threshold for insulin concentration during an OST for diagnosis of
EMS/ID is not appropriate in light of breed related differences. This work does fill a gap in knowledge by defining breed-specific ranges of insulin concentrations for evaluation of ID and also demonstrating that in general the insulin thresholds for ID are much lower than currently recommended. The present studies also determined that plasma glucose concentrations measured during an OST are not useful for diagnosis of ID, confirming earlier work. Therefore, recommendations for equine practitioners from this work would be:

1) Dynamic challenge tests are superior to baseline insulin concentrations for identifying horses that are IR.

2) Regarding interpreting or use of the OST:

   a. Insulin concentrations $\geq$ 22.8, 18.7, 30.2 or 26.3 $\mu$IU/mL, at 60, 75, 90 or 120 minutes respectively, are reasonable cut-offs to identify horses that are IR as defined by the FSGITT in most breeds.

   b. The OST appears to be a poor test for IR Morgans, if the OST is used in Morgan horses a stricter cut-off is required to identify ID/IR individuals.

   c. Breed specific cut-offs improve sensitivity and specificity of the OST for diagnosing IR. For example, an Arabian with an insulin value above 42 $\mu$IU/ml, and a WP with an insulin concentration above 26.3 $\mu$IU/ml at the 90 min time point are more likely to be ID/IR.

   d. Glucose concentrations during an OST are poor indicators of IR

3) Lack of obesity should not preclude diagnosis of a horse with ID and concern about EMS. And the ACVIM consensus statement [6] should be modified to say obesity and/or
regional adiposity may be present. Although certain breeds are predisposed to obesity (Arabians and WP), this study matched a horses or ponies body condition score (BCS) between the breeds so it was not significantly different and not a confounder.

With regard to the pathophysiology of EMS, this work is novel in that it marks the first time histological parameters in both adipose and muscle have been examined for correlations to insulin sensitivity and other measures of metabolic abnormalities (i.e. adipokines, etc.). Initial results suggest that, unlike humans, adipocyte size and muscle fiber type areas and proportions are not strongly correlated to SI or AIRg (and thus may not be important for diagnosing or characterizing horses with EMS/ID). However, this conclusion is based upon at least three possibly false assumptions (excluding not having a large enough sample size for muscle examination) with the first being that the FSIGTT and calculated minimal model parameters are a “gold standard” of diagnosing EMS/ID, an assumption that has been challenged recently with oral dynamic testing now considered more appropriate.[29] The second assumption is that the morphometrics assessed in the current study (area, proportion) are the best measurements for assessment of these tissues. To that end, evaluation of myocellular lipid infiltration and/or numbers of antigen presenting cells, or numer of mitochondria (to assess the metabolic potential of the adipocytes) may be better markers of the muscle and/or adipose dysfunction that occur in EMS/ID.[96,176,210,211] The third assumption is that the tissues sampled (middle gluteal muscle; tailhead subcutaneous adipose tissue) are representative of the ‘metabolic status’ of skeletal muscle and adipose tissue in general. However, depot differences exist in adipocyte size and inflammatory gene expression in horses,[96,110] and similarly, differences exist in skeletal muscle (fibers type, size and potentially gene expression) based on muscle group, site, and exercise (training)
Additional tissues (liver, pancreas) play a role in regulation of insulin and glucose dynamics, and histologic evaluation of these tissues may prove enlightening.

When evaluating breed differences in muscle and adipose tissue gene expression, this work has provided information to support future breed specific hypotheses regarding novel genes and networks of interest in the evaluation of metabolic function and dysfunction. This work represents the first time the entirety of the transcriptome of these two tissues has been examined and characterized in light of breed related differences in glucose and insulin dynamics.

Overall, the central hypothesis that “breed differences will result in variation of insulin dynamics, lipid metabolism, and the histologic and metabolic phenotype of skeletal muscle and adipose tissue” should be accepted in part. Breed differences were apparent in regards to insulin dynamics, lipid metabolism, adipocyte histology, and adipose and muscle metabolic (transcriptomic) parameters, but were not evident in gluteal muscle histology.

**Future Work**

**Dynamic Testing for Equine Metabolic Syndrome (EMS) and Insulin Dysregulation (ID)**

*Arginine Stimulation Test*

Future studies should examine more adult horses of the five breeds that formed the cohort for studies in this thesis and the associated sensitivities to insulin (based on responses to a FSIGTT and/or OST) to determine an insulin threshold for diagnosing ID or abnormal AIRg and to assess whether this threshold would differ by breed.
**Oral Sugar Test**

Future studies should examine whether different types of Karo syrup, with different compositions (some containing high fructose corn syrup), would elicit different insulin and glucose responses in different breeds of horses. Repeatability of the OST at the higher dose (0.25 ml/kg) could be performed. Further examination of insulin and glucose trajectory testing and correlations to other EMS traits, to assess whether diagnosis of EMS/ID can be refined should be pursued.

**Other Areas for General EMS/ID Testing**

Measurement of serum for additional biomarkers of EMS/ID such as \( IL6 \) and \( TNF \alpha \) can be performed in this cohort of animals. This would be undertaken to assess whether a greater sensitivity of EMS/ID diagnosis could be obtained by combining such biomarkers together with dynamic testing results.

Understanding the production and metabolism of non-esterified fatty acids (NEFAs) during a FSIGTT could be examined in this cohort of animals to better understand lipid metabolism differences either between breeds or between animals of high and low ranges of insulin sensitivities.

**Histology and EMS/ID**

Initial planned investigations include evaluation of a larger number of horses within each breed for muscle fiber type proportion and area analysis. Some of the other possible future directions to undertake in this area were alluded to in section 5.2. In brief, assessment of different depots for muscle and adipose, assessment of other tissues entirely (liver, pancreas),
and assessment for ectopic lipid accumulation and presence of antigen presenting cells, would all be areas worthy of future investigation. Additional areas of investigation would include comparing morphometric traits such as neck to withers height and neck circumference to girth circumference ratio to adipocyte size. Additionally, evaluating histologic features to OST outcome measures might prove more insightful. Investigation of succinate dehydrogenase levels, preferably in a quantitative manner, would help elucidate oxidative differences in the muscles of different horse breeds.

**Gene Expression Differences in Equine Muscle and Adipose Tissue and Their Relation to Systemic Markers and Phenotypes**

Initial planned investigations in this area include performing RNA Seq analysis on 62 more horses and/or ponies in muscle and adipose tissue samples to help increase our power to assess significant differences and to ensure our current conclusions remain valid after a larger analysis. The data obtained can then be evaluated in light of other EMS defining traits. For example, within this data set, though not described in this thesis, there are already a large number of genes that are differentially expressed when horses are grouped not by breed, but based on those horses/ponies that comprised the top 25% of high or low insulin responders or based on HMW adiponectin concentrations. While the transcriptome of these horses has been characterized, metabolomics on either serum or tissue can also be performed to obtain another level of understanding. One of the driving questions which initiated this investigation into breeds, was the role that myostatin might play in making QHs an insulin sensitive breed.[89,90,216] To that end, mechanistic studies using cell culture of equine myocytes or adipocytes with exposure to myostatin or myostatin and a myostatin inhibitor could be
performed to assess its effect on PPARC1 alpha and beta expression more directly. Evaluating QHs insulin and glucose responses to dynamic challenge tests, and tissue characteristics (histologic, transcriptomic, metabolomics, etc.) both in horses with and without the SINE insertion that affects myostatin expression could also be rewarding. Other cell culture studies could be performed on equine adipose tissue for example while altering some of the predicted upstream regulators, such as norepinephrine (which stimulates energy expenditure and local thermogenesis in brown and potentially beige adipose tissue),[217] to gain a more mechanistic understanding of how that would in fact influence transcript or protein expression downstream.

**Summarizing Statements**

In summary, this work has been transformative as, for the first time, aspects of metabolic function have been more fully characterized in several breeds of horses of differing susceptibilities to EMS/ID on multiple levels – systemic, histologic, and transcriptomic. This approach has enabled an increased understanding of breed susceptibility to EMS by elucidating knowledge of deeper levels of the tissue and molecular pathophysiology, and also has identified avenues for future hypotheses and investigations.
REFERENCES
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