Relationships between diet, obesity and insulin dysregulation in horses and ponies

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Abstract

Laminitis is a debilitating condition of equids that affects a significant proportion of domesticated horses and ponies (*Equus caballus*) worldwide. Prevention is the key to managing laminitis, as there are currently no effective treatments and crippling lameness can often necessitate the euthanasia of affected animals. The clinical clustering of obesity and insulin dysregulation as risk factors for laminitis has been referred to as equine metabolic syndrome. The studies reported in this thesis sought to further examine the relationships between diet, obesity and insulin dysregulation in horses and ponies.

Differences in the innate glucose and insulin dynamics of different equine breeds were established by investigating the insulin responses of animals to oral and intravenous glucose challenges. Ponies and Andalusian horses were relatively insulin resistant and hyperinsulinaemic compared with Standardbred horses; a finding that occurred without the potentially confounding effects of obesity or modifying dietary factors. Studies of diet-induced weight gain were then undertaken, in which a high dietary glycaemic load was found to influence the development of insulin dysregulation more than the induction of obesity *per se*. Relatively low plasma levels of adiponectin were identified in animals with reduced insulin sensitivity, whilst evidence of significant systemic inflammation was not detected. Glucagon-like peptide-1 (GLP-1) levels were found to correlate with postprandial insulin responses in horses and ponies adapted to cereal-rich meals.

These studies report for the first time the identification of innate differences in insulin metabolism between particular equine breeds. The induction of obesity was not associated with insulin dysregulation in horses and ponies fed a low glycaemic diet, suggesting that increased adiposity might be a consequence rather than a cause of insulin dysregulation in equids. Adiponectin may be a potentially useful biomarker for insulin dysregulation, although whether hypoadiponectinaemia is involved in the pathogenesis requires further investigation. Incretins such as GLP-1 could represent a potential therapeutic target for the control of equine hyperinsulinaemia.

Understanding how genetic predispositions to insulin dysregulation can be aggravated by the environment is an essential first step in the development of countermeasures to reduce the incidence of laminitis in equine populations worldwide.
Declaration

This is to certify that:

i. This thesis comprises only my original work towards the PhD except where indicated.

ii. Due acknowledgement has been made in text to all other material used.

iii. This thesis is fewer than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

Nicholas James Bamford
Preface

Publications forming part of this thesis


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Presentations arising from work in this thesis


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<td>American College of Veterinary Internal Medicine</td>
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<tr>
<td>AGE</td>
<td>Advanced glycosylation end-product</td>
</tr>
<tr>
<td>AIRg</td>
<td>Acute insulin response to glucose</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<td>BCS</td>
<td>Body condition score</td>
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<tr>
<td>BM</td>
<td>Body mass</td>
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<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BW</td>
<td>Body weight</td>
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<tr>
<td>CGIT</td>
<td>Combined glucose and insulin test</td>
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<tr>
<td>CNS</td>
<td>Cresty neck score</td>
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<tr>
<td>D₂O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DE</td>
<td>Digestible energy</td>
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<tr>
<td>DEXA</td>
<td>Dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>DI</td>
<td>Disposition index</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EHC</td>
<td>Euglycaemic-hyperinsulinaemic clamp</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EMS</td>
<td>Equine metabolic syndrome</td>
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FSIGT  Frequently sampled intravenous glucose tolerance test
GI  Glycaemic index
GIP  Glucose-dependent insulinotropic polypeptide
GLP-1  Glucagon-like peptide-1
GLUT  Glucose transporter
H₂O  Water
HDL-C  High-density lipoprotein-cholesterol
HMW  High molecular weight
IGF-1  Insulin-like growth factor-1
IL  Interleukin
IR  Insulin resistance
IRS  Insulin receptor substrates
IRT  Insulin response test
IV  Intravenous
LMW  Low molecular weight
\( M \)  Rate of glucose disposal calculated from the EHC
\( M/I \)  Insulin sensitivity index calculated from the EHC
MCP-1  Monocyte chemoattractant protein-1
MetS  Metabolic syndrome
MIRG  Modified insulin-to-glucose ratio
MMP  Matrix metalloproteinase
NEFA  Non-esterified fatty acid
NSC  Non-structural carbohydrate
<table>
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<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>OST</td>
<td>Oral sugar test</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator-inhibitor-1</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PPID</td>
<td>Pituitary pars intermedia dysfunction</td>
</tr>
<tr>
<td>QUICKI</td>
<td>Quantitative insulin sensitivity check index</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<tr>
<td>RISQI</td>
<td>Reciprocal of the square root of insulin</td>
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<tr>
<td>r_s</td>
<td>Spearman’s rank correlation coefficient</td>
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<td>SAA</td>
<td>Serum amyloid A</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>Sg</td>
<td>Glucose effectiveness</td>
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<tr>
<td>SI</td>
<td>Insulin sensitivity</td>
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<tr>
<td>TBFM</td>
<td>Total body fat mass</td>
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<tr>
<td>TBW</td>
<td>Total body water</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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Chapter 1 Literature review

1.1 General introduction

This thesis examines the relationships between diet, obesity and insulin dysregulation in horses and ponies (*Equus caballus*). There is growing recognition of a metabolic phenotype associated with insulin dysregulation in which seemingly healthy animals kept at pasture develop laminitis, a potentially fatal disease for which there is currently no effective treatment. The factors that are involved in the development of this metabolic phenotype require further investigation so that preventative countermeasures can be identified. This literature review will address current knowledge surrounding the link between metabolic disorders and laminitis, and outline the potential roles for diet and obesity in the modification of insulin dysregulation.

1.2 Laminitis

1.2.1 Background

The distal limb of ungulate species contains the unique anatomical structure known as the suspensory apparatus of the distal phalanx (Pollitt and Collins, 2016). The suspensory apparatus of the distal phalanx allows the transfer of weight from the appendicular skeleton to the hard, keratinised hoof wall by supporting the distal phalanx within the hoof capsule. Integral to this function are the lamellae, which form the strong interdigitating connections between the dermal attachment of the distal phalanx and the innermost epidermal layer of the hoof wall (Pollitt, 2010).

Pathological disruption of the lamellae results in the painful condition known as laminitis, leading to sinking or rotation of the distal phalanx within the hoof capsule (Hood, 1999). Laminitis represents one of the most important welfare concerns in domesticated equine populations due to the morbidity and mortality associated with the condition. Crippling lameness can result in the loss of functional locomotor activity and will sometimes necessitate the euthanasia of affected animals. The frequency of laminitis in domesticated equine populations has been estimated at between 1.5% and 34% (Wylie et al., 2011). The
wide range of these frequency estimates is reflective of the lack of high quality epidemiological data in populations that are not subject to selection bias.

Although much has been learned through studies of the pathophysiology of acute laminitis, the large knowledge gap still present makes it one of the most frustrating disease entities for veterinarians and horse owners alike (Moore and Belknap, 2009). The treatment of laminitis is limited once structural damage to the suspensory apparatus of the distal phalanx has occurred (Robertson et al., 2009). Therefore, the identification of risk factors for laminitis will enable the implementation of better management strategies that may help to reduce the incidence of clinical laminitis in equine populations (Harris et al., 2006).

1.2.2 Pathophysiology

Laminitis can be regarded as a systemic process that manifests as disease within the hoof capsule (Bailey et al., 2004). Although the suffix ‘-itis’ (denoting inflammation) is included in the terminology, it is now recognised that inflammation is not an important feature of all disease processes that ultimately lead to disruption of the lamellae. Recently, there have been four main mechanistic pathways proposed that result in the common end-point of laminitis (Katz and Bailey, 2012). These are: (1) inflammation and degradation of the lamellar extracellular matrix, (2) endothelial or vascular dysfunction, (3) endocrine or metabolic abnormalities and (4) mechanical overload. There may be some overlap in the pathophysiology of these proposed pathways, and further research is required to fully elucidate the relationships between the different aetiologies of laminitis.

The phases of laminitis can be broadly classified into predisposition, developmental (prodromal) and clinical stages. The predisposition stage occurs when animals are exposed to risk factors or ‘trigger factors’ that are associated with laminitis, such as underlying endocrine disease or the ingestion of rapidly fermentable carbohydrates (Harris et al., 2006). The developmental stage of laminitis is often undetectable; the pathological cascade that causes damage to the lamellae may take hours or days to become apparent. The clinical stage of laminitis can be further separated into acute, subacute and chronic phases. Acute laminitis is the classical presentation of lameness (often worse in the forelimbs due to the extra carriage of bodyweight), bounding digital pulses and increased hoof wall temperature (Pollitt, 2004). The Obel grading system is used to assess the severity of acute laminitis, with
lameness scored on a scale of 0 to 4 (Obel, 1948). The subacute stage is often associated with mild or undetected episodes of clinical laminitis, where progressive structural damage to the lamellae will affect normal hoof growth and eventually lead to chronic lameness.

Irreversible damage to the lamellae has already occurred by the time naturally-occurring cases of laminitis are presented with lameness to veterinarians. Therefore, experimental models have been developed to allow researchers to study the prodromal stage of laminitis under controlled conditions. These models include the black walnut extract model, the carbohydrate overload models (using starch or oligofructose) and the hyperinsulinaemic model (Katz and Bailey, 2014). A limitation of all experimental models is that large lamellar biopsy samples for histopathology can only be obtained post mortem and are therefore only representative of a single time-point during a dynamic process. Small biopsy samples can be taken during an experimental procedure, but they may cause additional pain, inflammation and disruption of the lamellar architecture in the region from which they are collected. Studies have therefore had to consider the time of euthanasia carefully to avoid the excessive use of experimental animals. Furthermore, it is not clear how representative each model is of the development of naturally-occurring disease.

**1.2.3 Inflammation and laminitis**

Laminitis is a well-recognised clinical sequel to systemic inflammation (sepsis) that is associated with conditions such as alimentary grain overload, colitis, metritis and pleuropneumonia (Geor and Frank, 2009). The role of inflammation in the development of laminitis has been studied using both the black walnut extract experimental model and the carbohydrate overload experimental models. The administration of black walnut extract to horses causes an overwhelming systemic inflammatory response, including the induction of severe laminitis (Belknap, 2010). The causative agent within black walnut extract is still unknown. This induction model is characterised by the rapid upregulation of proinflammatory cytokines and neutrophil infiltration within the lamellae (Black et al., 2006; Belknap et al., 2007). The carbohydrate overload models also lead to profound systemic inflammation, but lamellar damage appears to be later in onset (Belknap and Black, 2012). Similar to the lamellar changes observed with the black walnut extract model, cytokines upregulation and leukocyte infiltration are a feature (Faleiros et al., 2011; Leise et al., 2011).
Matrix metalloproteinase (MMP) activity is also upregulated within the lamellae of horses subjected to both experimental models (Loftus et al., 2009). Although it was thought that degradation of the basement membrane by MMP-2 and MMP-9 was an important mechanistic process, it is contended that this might actually be a consequence of neutrophil emigration rather than an intrinsic cause of laminitis (Katz and Bailey, 2014). Hypoxia-inducible factor-1α (HIF-1A) plays an important regulatory role in inflammatory gene expression in many tissues, with evidence that this protein is upregulated in the lamellae of horses subjected to sepsis-related models of laminitis (Pawlak et al., 2014).

Further discussion of the pathophysiology of inflammatory laminitis is beyond the scope of this literature review. Instead, the role of endocrine or metabolic abnormalities in the pathogenesis of equine laminitis will be highlighted.

1.2.4 Endocrine disorders and laminitis

Endocrine and metabolic disorders have been anecdotally regarded as the most common cause of laminitis cases presented to veterinarians (Geor and Frank, 2009). In one hospital-based equine population, almost 90% of laminitis cases demonstrated evidence of an endocrinopathy in the absence of other systemic illness (Karikoski et al., 2011). Endocrinopathic laminitis is often insidious in onset and affected animals may experience repeated mild bouts of clinical disease before it is appropriately recognised by owners (Karikoski et al., 2015). Chronic, progressive damage to the suspensory apparatus of the distal phalanx may mean that each episode of clinical laminitis will become more severe until humane euthanasia of the animal is indicated. Investigations of the endocrine and metabolic status of animals affected by this form of laminitis have identified hyperinsulinaemia to be a common feature (Jeffcott et al., 1986; Frank et al., 2006; Treiber et al., 2006; Bailey et al., 2008). Furthermore, hyperinsulinaemia has been correlated with laminitis severity (McGowan et al., 2004; Walsh et al., 2009) and was predictive of future episodes of laminitis in animals with basal insulin concentrations greater than 32 mIU/L (Carter et al., 2009c).

In order to investigate the role of hyperinsulinaemia in the pathogenesis of laminitis, researchers developed an experimental induction model using a prolonged euglycaemic-hyperinsulinaemic clamp (EHC) technique (de Laat et al., 2010a). During the procedure, insulin is infused at a constant rate to maintain serum insulin concentrations at greater than
1000 mU/L, whilst glucose is concurrently infused at a variable rate to maintain normal blood glucose concentrations of approximately 5.0 mmol/L. Application of the modified EHC procedure to healthy ponies (Asplin et al., 2007) and Standardbred horses (de Laat et al., 2010b) resulted in the development of clinical laminitis in all animals after 48 hours. These studies suggest that there is a direct role for hyperinsulinaemia in the pathogenesis of laminitis associated with endocrine and metabolic abnormalities. The pathophysiology of insulin-induced laminitis is not yet fully understood; however, there are several different theories that have been described.

The hallmark histological features of insulin-induced laminitis include abnormal keratinisation and increased mitotic activity, with lengthening of the secondary epidermal lamellae (Asplin et al., 2010; de Laat et al., 2013a; Karikoski et al., 2014). Unlike the inflammatory models of laminitis there is minimal cytokine or MMP activity (de Laat et al., 2011; Burns et al., 2015). The histology of insulin-induced laminitis is suggestive of a mitogenic effect of insulin, which is supported by the observation that lamellar epithelial cells proliferate when incubated with insulin in vitro (Bailey and Chockalingham, 2010). However, insulin receptors are not present on the surface of lamellar epithelial cells (Burns et al., 2013); it may therefore be the case that insulin exerts its effects through insulin-like growth factor-1 (IGF-1) receptors which are present on the surface of lamellar epithelial cells (Burns et al., 2013; de Laat et al., 2013b).

Glucose deprivation secondary to marked insulin resistance has been mentioned as a possible mechanism due to the high glucose requirement of lamellar epithelial cells (Pass et al., 1998). However, it has been shown that glucose uptake in the lamellae occurs through insulin-independent transporters (Asplin et al., 2011). Glucotoxicity has also been mentioned as another glucose-related process that could result in lamellar failure (Johnson, 2002; Katz and Bailey, 2012). The accumulation of advanced glycosylation end-products (AGE) in cells during sporadic or persistent hyperglycaemia is known to damage tissues, as seen in human diabetic patients (Stumvoll et al., 2005). However, horses and ponies with insulin resistance are rarely hyperglycaemic. When samples from animals that developed laminitis during the modified EHC procedure were reviewed, AGE did not accumulate in lamellar tissues during the developmental stages (de Laat et al., 2012a). An experimental model that further studied the effect of hyperglycaemia by infusing glucose in the absence of exogenous insulin was able to induce laminitis in healthy horses after 48 hours (de Laat et al., 2012b). However, it
is difficult to separate the pathophysiological effect of hyperglycaemia from the endogenous hyperinsulinaemia that also occurred under such conditions.

The effect of glucotoxicity might not be directly on the lamellae. It may be that glucotoxicity is a contributing factor to the development of endothelial dysfunction within the digital vasculature (Frank and Tadros, 2014). In human diabetic patients, glucotoxicity is known to cause a decrease in nitric oxide (a vasodilator) production and an increase in endothelin-1 (a vasoconstrictor) production to favour a vasoconstrictive state (Garcia et al., 2010). Under normal conditions, insulin exerts a vasodilatory effect by inducing the release of nitric oxide from the endothelium. The state of insulin resistance that is likely to occur during hyperinsulinaemia could also lead to alterations in blood flow through the lamellar tissues. Dysfunction of the numerous arteriovenous anastomoses present in the digital circulation has been proposed as a mechanism through which alterations in blood flow may contribute to the development of laminitis (de Laat et al., 2010a). When incubated with high concentrations of insulin in vitro, equine digital vessels have been shown to lose the ability to dilate (Venugopal et al., 2011). Additionally, in vivo studies have demonstrated systemic hypertension in insulin resistant ponies (Bailey et al., 2008). Further investigation of the role of vascular endothelial dysfunction in the development of insulin-induced laminitis is required.

1.2.5 Pituitary pars intermedia dysfunction and laminitis

Pituitary pars intermedia dysfunction (PPID; equine Cushing’s disease) is the most common endocrinopathy of equids aged over 15 years (McFarlane, 2011). This neurodegenerative condition is characterised by the progressive loss of dopaminergic inhibitory neurons leading to melanotrope hypertrophy and micro- or macroadenoma formation within the pars intermedia of the pituitary gland (Schott, 2002). Laminitis is an important clinical feature of PPID, with approximately 13% of affected animals displaying evidence of active or previous laminitis (McGowan et al., 2013). The link between PPID and laminitis was initially proposed to be the result of hypercortisolaemia; however, there is no evidence to support such a link and hyperinsulinaemia is now recognised to be the important abnormality that is associated with the development of laminitis in these animals (McFarlane, 2014). Hyperinsulinaemia has been shown to be predictive of future episodes of laminitis, correlated
with disease severity and prognostic of medium-term survival in animals with PPID (McGowan et al., 2004; Walsh et al., 2009).

1.2.6 Pasture-associated laminitis

The term ‘pasture-associated laminitis’ has been used to describe cases of laminitis that occur in seemingly healthy horses and ponies grazing pasture; and in the absence of systemic inflammatory disorders such as colitis, duodenitis/proximal jejunitis, metritis and pleuropneumonia (Geor, 2010). Epidemiological studies have reported that between 45% and 60% of equine laminitis cases occur under these circumstances (Hinckley and Henderson, 1996; USDA, 2000). One study followed a cohort of horses and ponies kept at pasture for several years, and reported that approximately 23% of the entire population suffered from at least one episode of laminitis (Menzies-Gow et al., 2010a). The pathophysiology of pasture-associated laminitis is not fully understood and is likely to be multifactorial (Katz and Bailey, 2012).

Episodes of pasture-associated laminitis most often occur when conditions favour the accumulation of fructans in grasses (Longland and Byrd, 2006; Menzies-Gow et al., 2010a). It is thought that pasture can contain sufficient quantities of fructan carbohydrate that might lead to disturbances in the hindgut microflora of horses (Crawford et al., 2007). As mentioned previously, the enteral administration of oligofructose is one of the experimental models used to study acute laminitis (van Eps and Pollitt, 2006). Although this model may provide a link between pasture fructan consumption and lamellar failure, it is not clear how well it reflects the pathogenesis of naturally occurring pasture-associated laminitis. It has been questioned whether horses and ponies at pasture are able to voluntarily ingest an equivalent amount of fructan in a short enough period of time (Harris et al, 2006). Additionally, the severe gastrointestinal disturbances observed during the oligofructose induction model are not a feature of naturally-occurring pasture-associated laminitis (Bailey et al., 2009). It is also important to note that only certain animals develop laminitis when large groups of horses and ponies graze pasture under the same conditions. In fact, certain individuals appear predisposed to recurrent episodes of laminitis whilst other animals never suffer from laminitis (Menzies-Gow et al., 2010a). This might mean that a direct role for oligofructose-induced hindgut disturbances in pasture-associated laminitis is less likely.
Hyperinsulinaemia is a recognised risk factor for laminitis in horses and ponies with underlying endocrine or metabolic disorders (McGowan, 2010). The exacerbation of hyperinsulinaemia through the ingestion of soluble carbohydrates in pasture may therefore be a plausible pathway through which laminitis occurs in susceptible horses and ponies. One study demonstrated that previously-laminitic ponies had exaggerated insulin responses after feeding inulin (a type of fructan carbohydrate) when compared with control ponies (Bailey et al., 2007). Furthermore, it has been recognised that there is an endocrine or metabolic phenotype that is distinct from PPID in equids that are predisposed to pasture-associated laminitis (Geor, 2010). These animals are often, although not always, obese and display hyperinsulinaemia or evidence of insulin resistance (Johnson, 2002; Frank et al., 2006; Treiber et al., 2006; Carter et al., 2009c). It is the investigation of this phenotype that has attracted the majority of recent attention.

1.3 Equine metabolic syndrome

1.3.1 Background

Equine metabolic syndrome (EMS) is the term that is used to describe the endocrine and metabolic phenotype of certain horses and ponies that are predisposed to pasture-associated laminitis (Frank et al., 2010). The condition was first described by Johnson (2002) and has been variously referred to as peripheral Cushing’s disease, equine insulin resistance syndrome, pre-laminitic metabolic syndrome and syndrome X (Kronfeld, 2003; Treiber et al., 2006). The wide acceptance of EMS terminology has arisen from the purported similarity between certain features of EMS and the metabolic syndrome (MetS) that has been described in humans. Metabolic syndrome describes human patients with a collection of certain risk factors that are associated with the development of cardiovascular disease and type 2 diabetes mellitus (Fulop et al., 2006; Alberti et al., 2009). Insulin resistance is a central component of both EMS and MetS, with other clinical abnormalities including generalised obesity and regional adiposity, dyslipidaemia and hypertension (Table 1-1).

The unique feature of EMS is the predisposition of affected animals to laminitis. Type 2 diabetes mellitus has been reported in horses, and may be an under-recognised condition, but the equine pancreas rarely succumbs to β cell exhaustion (Durham et al., 2009). Therefore, unlike MetS where cardiovascular disease and type 2 diabetes mellitus are potentially fatal
1.3.2 Consensus statement

The central features of EMS were defined in a consensus statement of the American College of Veterinary Internal Medicine (ACVIM; Frank et al., 2010). Elements that were considered essential to the definition of EMS in the majority of affected equids were: (1) generalised obesity or increased regional adiposity, (2) evidence of insulin resistance, hyperinsulinaemia or exaggerated insulin responses to oral glucose, and (3) a predisposition toward laminitis. Additional components of the syndrome that were proposed to warrant further investigation included hypertriglyceridaemia or dyslipidaemia, hyperleptinaemia or leptin resistance, arterial hypertension, abnormal reproductive function and systemic inflammation (Frank et al., 2010).

There is an argument that laminitis should not form part of the definition of EMS, since the recognition of these phenotypic characteristics should allow the identification of ‘at risk’ animals before clinical laminitis is apparent. There is also some controversy as to whether obesity is an essential component of EMS. Although many horses and ponies with hyperinsulinaemia and insulin resistance are obese, endocrinopathic laminitis is recognised in non-obese horses and ponies (Bailey et al., 2007; Borer et al., 2012b). Conversely, obese horses and ponies are not necessarily insulin resistant or predisposed to laminitis (Geor, 2010). Insulin is an anabolic hormone and it may therefore be that obesity is a consequence rather than a cause of insulin resistance. More recently, obesity has been discussed as a modifying factor that may amplify the potentially harmful effects of hyperinsulinaemia in affected horses and ponies (Frank and Tadros, 2014).

1.3.3 Genetics and breed differences

It has long been recognised that certain breeds of horses (e.g. Morgans, Paso Finos, Spanish Mustangs, Tennessee Walking Horses, European warmbloods) and ponies appear to be more susceptible to EMS (Johnson, 2002; Geor, 2010; Frank, 2011). These animals have often been referred to as ‘easy keepers’ due to their apparently high metabolic efficiency. Different
equine breeds almost certainly evolved under diverse selection pressures that will have influenced a wide range of metabolic processes; there is evidence that pony breeds and Spanish horses developed from different evolutionary lines to Thoroughbred and Standardbred horses (Jansen et al., 2002). Studies of comparative physiology have demonstrated that insulin resistance is a common metabolic strategy to promote the storage of body fat in mammalian species that undergo hibernation, migration or seasonal changes in the availability of forage (Johnson et al., 2013). Ponies appear to have retained a degree of this adaptation, as demonstrated by seasonal changes in metabolic rate, appetite and body mass (Dugdale et al., 2011a; Brinkmann et al., 2012).

The concept of a ‘thrifty genotype’ was first proposed by Neel (1962) to explain why certain people have a higher metabolic efficiency than others. Thrifty genes may influence the metabolism of equids too. Treiber et al. (2006) demonstrated a dominant pattern of inheritance for the incidence of laminitis in an inbred herd of Dartmoor and Welsh ponies kept at pasture. A study of pasture-associated laminitis in the United Kingdom also showed that ponies were more than twice as likely to develop pasture-associated laminitis as larger breed horses (Menzies-Gow et al., 2010a). A genetic predisposition to disease is a reasonable hypothesis for these observations, although polymorphisms in multiple genes are more likely in the wider equine population. It has been postulated that an interaction between the thrifty genotype and modern environmental conditions, such as improved pastures, has resulted in the absence of seasonal weight loss, progressive obesity, and the exacerbation of insulin resistance in domesticated equids (Kronfeld et al., 2005b). The way in which the environment can influence the expression of different genes is referred to as ‘epigenetics’ (Jaenisch and Bird, 2003).

1.4 Insulin dysregulation

1.4.1 Definition

Insulin dysregulation is a recently-proposed term that refers to insulin resistance, hyperinsulinaemia and/or excessive insulin responses to oral carbohydrates (Frank and Tadros, 2014). This umbrella term encompasses all aspects of abnormal insulin metabolism that can be difficult to separate from each other because of variations in terminology, definition and methods of detection (Kronfeld et al., 2005b).
1.4.2 Insulin physiology

Insulin is a peptide hormone secreted by the β cells of the islets of Langerhans within the pancreas. The classical action of insulin is to maintain normoglycaemia by stimulating glucose uptake in skeletal muscle, liver and adipose tissue, and suppressing hepatic gluconeogenesis (Magkos et al., 2010). Additionally, insulin has important pleotropic effects including the regulation of protein, carbohydrate and lipid metabolism; the anabolic promotion of cell growth and division; and the modulation of vascular smooth muscle tone (Schmidt and Hickey, 2009). Insulin is formed as a prohormone and packaged by the Golgi apparatus into secretory granules. Proinsulin consists of A and B chains that are linked by disulphide bonds. The cleavage of a connecting peptide (C-peptide) by endopeptidases is the final process in the formation of the biologically active dipeptide of insulin within secretory granules.

Insulin secretion is biphasic, characterised by the initial rapid release of preformed insulin followed by a slower, more sustained, release of both preformed and newly synthesised insulin that is proportional to the degree of glycaemia (Wilcox, 2005; Vervuert et al., 2009). Postprandial glucose absorption is the primary stimulus for insulin secretion, but there are a range of stimuli that promote the release of insulin, including acetylcholine (parasympathetic nervous system), gastrointestinal hormones such as secretin and cholecystokinin, specialised incretin hormones and amino acids such as arginine. Inhibitory mediators include catecholamines (sympathetic nervous system), corticosteroids and hormones such as somatostatin.

The movement of glucose into peripheral tissues occurs through transmembrane glucose transporter (GLUT) glycoproteins. Five main types of GLUT glycoproteins exist, varying in their distribution, affinity for glucose and dependency on insulin. The insulin-independent GLUT1 is present within the cell membranes of all tissues and facilitates basal cellular glucose supply (Wilcox, 2005). The insulin-dependent GLUT4 is responsible for the majority of whole-body glucose disposal, primarily into skeletal muscle (60-70%) and adipose tissue (10%) (Smith, 2002). Although the liver does not rely solely on insulin-mediated glucose update, it does account for up to 30% of whole-body insulin-mediated glucose disposal. The binding of insulin to its receptor on the cell surface results in the autophosphorylation of tyrosine kinase and the downstream phosphorylation of insulin-receptor substrates (IRS).
These substrates facilitate the translocation of GLUT4 from the cytoplasmic pool to the cellular membrane via phosphoinositide 3-kinase (PI3-K) activity (Saltiel and Khan, 2001).

The facilitation of cellular glucose uptake by insulin is accompanied by the activation of pathways that favour energy storage (Magkos et al., 2010). These pathways lead to the inhibition of gluconeogenesis, glycogenolysis and lipolysis; and the promotion of glycogen and triglyceride synthesis and storage. The anabolic effects of insulin, including cell proliferation and protein synthesis, are mediated by mitogen activated protein kinase (MAPK) pathways (Saltiel and Khan, 2001). Insulin also plays an important role in vascular function through its endothelial-dependent vasodilatory effects that are mediated by the release of nitric oxide (Muniyappa et al., 2008).

1.4.3 Insulin resistance

Insulin resistance can be defined as the attenuation of an appropriate physiological response to normal or elevated levels of insulin; the classical manifestation of insulin resistance is impaired insulin-mediated glucose disposal (Wilcox, 2005). Hyperinsulinaemia is a normal adaptive response to reduced insulin sensitivity; therefore, insulin resistance and hyperinsulinaemia are inherently related as long as the pancreas maintains the ability to synthesise and release insulin (termed ‘compensated’ insulin resistance). If pancreatic insulin secretion is not proportional to a given degree of insulin resistance, this is termed ‘uncompensated’ insulin resistance (Kronfeld et al., 2005b).

Hyperinsulinaemia in horses may occur as a result of increased insulin secretion, decreased hepatic insulin clearance, or both (Toth et al., 2010). The study of insulin secretion and clearance relies on the measurement of C-peptide concentrations, because C-peptide is released from secretory granules in equimolar amounts to insulin but is not subjected to rapid hepatic clearance. The measurement of C-peptide is therefore reflective of secretion and can be used to model the removal of insulin from the bloodstream by the liver (Frank and Tadros, 2014). Insulin resistance is believed to occur at the cellular level through a combination of receptor down-regulation and post-receptor signal impairment (Piya et al., 2013). It does not appear to be total GLUT4 protein content, but rather processes relating to intracellular GLUT4 trafficking that are impaired in insulin resistant horses (Waller et al., 2011).
The consequences of insulin dysregulation in horses that pertain to the pathogenesis of laminitis have been mentioned previously. The principle effect of hyperinsulinaemia *per se* appears to be related to the mitogenic effect of insulin on lamellar epithelial cells (Asplin et al., 2010; Bailey and Chockalingham, 2010; de Laat et al., 2013a; Karikoski et al., 2014). Vascular endothelial insulin resistance may lead to a decrease in nitric oxide-mediated vasodilation and increase in endothelin-1-mediated vasoconstriction (Garcia et al., 2010), potentially altering digital and lamellar blood flow (Katz and Bailey, 2012). One study has reported systemic hypertension in insulin resistant ponies (Bailey et al., 2008).

1.4.4 Insulin-like growth factor-1

Insulin-like growth factor-1 is a protein hormone that shares a high degree of structural homology with insulin (Laviola et al., 2007). It is a primary effector of growth hormone that is released in large quantities by the liver, but also in smaller quantities by various tissues to exert local paracrine effects. The main function of IGF-1 is to regulate cellular growth and repair in almost every tissue of the body (Adams et al., 2000). The IGF-1 receptor is a tyrosine kinase from the same family as the insulin receptor that exert their cellular effects through the activation of PI3-K pathways. Due to the similarities between insulin and IGF-1, there is some cross-over between these hormones and their receptors, albeit with much less reciprocal affinity and lower post-binding potency. This is an important consideration in the pathogenesis of hyperinsulinaemic laminitis. As mentioned previously, insulin may exert its mitogenic effects on the lamellar epithelial cells by binding to IGF-1 receptors (Burns et al., 2013; de Laat et al., 2013b).

1.5 Assessment of insulin sensitivity

Insulin sensitivity can be assessed using number of different testing protocols, all of which have inherent advantages and limitations. The primary determinants of glucose and insulin dynamics are: (1) the insulin secretory response and (2) the sensitivity of skeletal muscle and adipose tissue to the effects of insulin (Firshman and Valberg, 2007). Tests can be divided into non-specific indicators or quantitative measures of insulin sensitivity (Kronfeld et al., 2005a). A major limitation of the most accurate quantitative tests that are used to assess
insulin sensitivity is their impracticality beyond the research setting (Geor, 2008). Therefore, several surrogate tests (both resting and dynamic) have been described in horses that are more appropriate to a clinical setting. Many of these tests require further investigation to optimise test performance and specific cut-offs to define laminitis risk are often lacking (Frank, 2011).

1.5.1 Basal measurements

Non-specific indicators of insulin dysregulation include the measurement of resting glucose and insulin concentrations. These measurements suffer from an inherent lack of sensitivity and specificity due to the potential for external factors (such as stress and feeding status) to influence single measurements (Tiley et al., 2007). Hyperinsulinaemia is an expected physiological response to peripheral insulin resistance and may therefore be a simple and specific indicator. However, resting hyperinsulinaemia is considered to be a weak means of detecting insulin resistance due to a lack of sensitivity (Kronfeld et al., 2005a). A suggested cut-off to define resting hyperinsulinaemia in horses is a fasting insulin concentration of 20 mU/L (Frank et al., 2010). Frank and Tadros (2014) have asserted that many horses and ponies with insulin dysregulation will only demonstrate hyperinsulinaemia during dynamic testing protocols such as an oral or intravenous carbohydrate challenge.

Fasting hyperglycaemia is not commonly detected in horses with insulin dysregulation, although the routine measurement of basal glucose concentrations is recommended to screen for uncompensated insulin resistance or diabetes mellitus (Frank, 2011). Fructosamine, a type of glycated amine that forms during periods of hyperglycaemia, can be used as a surrogate marker of short term glycaemic control (Johnson et al., 1983). Increased fructosamine concentrations were detected in previously-laminitic horses when compared with normal horses (Knowles et al., 2012), although the previously-laminitic group was not comprised exclusively of horses with EMS. The measurement of fructosamine is becoming less popular in human medicine and is not currently recommended in the routine evaluation of horses and ponies with suspected insulin dysregulation.

Proxy measurements of insulin sensitivity have been extrapolated from human studies and applied to horses (Treiber et al., 2005b; Borer et al., 2012a). These parameters are calculated using basal glucose and insulin concentrations in an effort to improve the sensitivity and specificity of using basal concentrations alone. The proxies that performed the best when
compared to quantitative assessments of insulin sensitivity horses included the reciprocal of
the square root of insulin (RISQI), the quantitative insulin sensitivity check index (QUICKI)
and the modified insulin-to-glucose ratio (MIRG), which were calculated as follows:

\[
RISQI = \text{insulin concentration}^{-0.5}
\]

\[
QUICKI = \frac{1}{\log[\text{insulin concentration}] + \log[\text{glucose concentration}]}
\]

\[
MIRG = \frac{(800 - 0.3 \times [\text{insulin concentration} - 50]^2)}{\text{glucose concentration} - 30}
\]

Proxy measurements may provide an approximation of the quantitative methods used to
assess insulin sensitivity; however, care must be taken when interpreting results due to the
use of potentially labile glucose and insulin concentrations. Proxy measurements may be a
useful screening test in large population-based studies, but the use of dynamic tests are
preferred for the assessment of insulin dysregulation in individual animals (Frank et al.,
2010).

1.5.2 Quantitative methods

Methods that quantitatively assess glucose and insulin dynamics are considered to be the
‘gold standard’ (Rijnen and van der Kolk, 2003). As mentioned previously, the use of these
tests is largely restricted to use in research studies due to impracticality in the clinical setting.
The two tests of this nature that have been used in equine studies include the euglycaemic-
hyperinsulinaemic clamp (EHC) and the insulin-modified frequently-sampled intravenous
glucose tolerance test (FSIGT) (Bergman et al., 1979; Kronfeld et al., 2005a).

As the name suggests, the EHC procedure involves the induction and maintenance of
hyperinsulinaemia using a continuous intravenous infusion of insulin, during which a variable
glucose infusion is used to maintain normoglycaemia (Pratt et al., 2005). After reaching a
steady state, the rate of glucose infusion will be equal to the rate of glucose uptake. The
parameters routinely calculated from the EHC are: (1) rate of whole-body glucose uptake
\((M)\), expressed as units of mmol/kg/min; and (2) insulin sensitivity index \((M/I)\), which is
calculated as the ratio of glucose uptake relative to the insulin concentration \((I)\) and expressed
as units of mmol/kg/min\(\cdot\)mIU/L\(^{-1}\). This procedure is technically difficult, requires
specialised equipment (including precise infusion pumps) and has been criticised by some for
being a non-physiologic measurement of insulin sensitivity due to the hyperinsulinaemia induced (Kronfeld et al., 2005a).

The successful application of a FSIGT to horses was first reported by Hoffman et al. (2003) and has been more commonly utilised in equine studies than the EHC. This procedure involves the collection of baseline blood samples at -60, -45 and 0 minutes prior to the administration of a glucose bolus (300 mg/kg BW) intravenously. Blood samples are collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16 and 19 minutes post-infusion. A dose of insulin (20 mIU/kg BW) is then given intravenously with further blood samples collected at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 minutes. Some investigators extend the sampling period to as long as 360 minutes to ensure a return to baseline values in insulin resistant animals (Bailey et al., 2007). Plasma samples are analysed to yield both glucose and insulin concentrations over the testing period. A modification of the FSIGT procedure has been described, in which a lower dose of dextrose (100 mg/kg BW) is used to minimise the urinary spilling of glucose (Toth et al., 2009).

The glucose and insulin curves generated using the FSIGT are analysed using specialised computer software (MINMOD Millennium, University of Pennsylvania, Kennett Square, Pennsylvania, USA) as described by Boston et al. (2003). The algorithm output includes: (1) insulin sensitivity (SI), which quantifies the ability of insulin to promote glucose disposal and inhibit glycolysis, is calculated as the change in glucose per unit change in insulin and expressed as units of L/(mIU·min); (2) glucose effectiveness (Sg), which quantifies the ability of glucose to mediate its own disposal, is calculated as the change in glucose independent of insulin and expressed as units of min⁻¹; (3) acute insulin response to glucose (AIRg), which quantifies the pancreatic insulin response to the glucose challenge, is calculated as the area under the insulin curve during the first 10 minutes of the FSIGT and expressed as units of (mIU·min)/L; and (4) disposition index (DI), which is indicative of the appropriateness of the insulin response for a given level of insulin sensitivity, is calculated as the multiplication product of SI x AIRg and expressed as a numeric value without units.

1.5.3 Dynamic intravenous tests

In order to simplify the testing procedures described above, several dynamic intravenous tests have been described for use in horses. These tests aim to provide a balance between...
practicality and accuracy in a clinical setting. A combined glucose and insulin test (CGIT) has been developed for horses (Eiler et al., 2005). This procedure involves the administration of a glucose bolus (150 mg/kg BW) immediately followed by an insulin dose (0.1 IU/kg BW) intravenously, with the collection of sequential blood samples at 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135 and 150 minutes. Various analyses can be used to interpret the CGIT, including area under the glucose curve, area under the insulin curve and the time taken for blood glucose to return to baseline values (Frank et al., 2006). A minimalist version of this procedure can involve the collection of blood samples at baseline and 45 minutes for the measurement of glucose and insulin (Morgan et al., 2016). Insulin resistance is suggested when blood glucose concentrations fail to normalise by 45 minutes. A distinction between compensated and uncompensated insulin resistance can also be determined, depending on whether insulin levels remain high (>100 mIU/L) at 45 minutes. The use of this test as a clinically-useful surrogate for the more rigorous quantitative tests has been supported by the ACVIM (Frank et al., 2010).

The insulin response test (IRT) utilises an intravenous insulin challenge only, followed by the measurement of blood glucose concentrations. A dosage regimen of 100 mIU/kg BW has been reported (Eiler et al., 2005; Caltabilota et al., 2010). Glucose curves can be generated by collecting blood samples at 5, 15, 30, 45, 60, 90, 120, 150 and 180 minutes following the insulin injection, with the time to reach 50% baseline glucose concentrations used in the interpretation of this test. More recently, a two-step IRT has been described, in which blood glucose is measured only at baseline and 30 minutes after the insulin injection (Bertin and Sojka-Kritchevsky, 2013). A reduction of blood glucose concentrations below 50% of baseline is used to discriminate between insulin sensitive and insulin resistant horses. A theoretical risk of this procedure is the development of severe hypoglycaemia in insulin sensitive horses, although this was not appreciated during experimental studies (Caltabilota et al., 2010; Bertin and Sojka-Kritchevsky, 2013). The prophylactic use of dextrose solutions after 30 minutes can be used to mitigate this potential complication.

1.5.4 Dynamic oral tests

Exaggerated insulin responses to oral carbohydrates is an important characteristic of insulin dysregulation in horses and ponies (Frank and Tadros, 2014). Therefore, the use of field tests
that evoke an insulinaemic response using an oral glucose challenge are becoming more popular with both researchers and clinicians, especially because basal hyperinsulinaemia is so insensitive for the diagnosis of insulin dysregulation. Several oral glucose tolerance test (OGTT) methodologies have been applied to horses (Firshman and Valberg, 2007). It is important to note that factors such as the rates of glucose intake, gastric emptying and intestinal absorption will influence the glycaemic response, whilst factors such as insulin sensitivity, pancreatic β cell capacity and the action of insulin secretagogues will influence the insulinaemic response (Kronfeld et al., 2005a).

The traditional OGTT involves the administration of dextrose (typically 1.0 g/kg BW) via nasogastric tube, which has been used to evaluate horses with suspected intestinal malabsorption as well as in the evaluation of glucose and insulin dynamics (Jeffcott et al., 1986; Mair et al., 1991). Due to the difficulty in passing a nasogastric tube in some animals, and the potential for stress to affect insulin sensitivity, an in-feed OGTT protocol has also been described. This method requires the horse or pony to voluntarily consume a fibre-based (chaff) meal containing added glucose (Borer et al., 2012b). Serial blood samples can be collected at various time-points to measure glucose and insulin concentrations. Peak glucose and insulin concentrations, time to peak concentrations and area under the respective curves (AUC) are the most commonly calculated variables (Vervuert et al., 2009). One reference interval that is widely used for normal horses that undergo an in-feed OGTT (1.0 g/kg BW dextrose) is an insulin concentration of <87 mU/L at the 2-hour blood sample (Durham, unpublished data).

The OST has been advocated as a more user-friendly version of an OGTT that has gained widespread acceptance in North America due to the availability of a commercial corn syrup product (Karo light syrup) from most supermarkets (Frank, 2012). This test involves the oral administration of corn syrup (15 ml/100 kg BW) to provide 150 mg/kg BW of dextrose-derived soluble sugars (Schuver et al., 2014). The reference range for normal horses is an insulin concentration of <60 mU/L at 60 to 90 minutes after the administration of corn syrup (Frank, 2011). Oral sugar test results were correlated ($r_s=0.90$) with FSIGT results in one study (Schuver et al., 2014), but were poorly correlated ($r_s=0.13$) with EHC results by other investigators (Banse and McFarlane, 2014). This simple test may hold promise for the practical diagnosis of insulin dysregulation in the field, but further test validation in a larger number of horses would be of benefit.
1.6 Obesity

1.6.1 Background and epidemiology

Obesity is defined by the World Health Organisation (WHO) as abnormal or excessive fat accumulation that may impair health\(^1\). Mirroring the alarming rates of obesity in human populations, companion animals including dogs and cats are suffering from obesity in high numbers (German, 2006). Horses are not immune to the obesity epidemic, which is considered by some to be one of the most important welfare issues of modern times (Sillence et al., 2006; Owers and Chubbock, 2013). Survey studies have confirmed a high prevalence of obesity among certain equine populations, with reports of between 27 and 50% of horses being considered over-conditioned or obese (Wyse et al., 2008; Thatcher et al., 2012; Giles et al., 2014). Notably, one study in the United Kingdom found higher body condition to be associated with laminitis severity and non-survival in horses and ponies suffering from pasture-associated laminitis (Menzies-Gow et al., 2010b).

Adiposity (amount of stored body fat) can be considered most simplistically as a reflection of the balance between energy intake and expenditure. The development of obesity in horses most often results from chronic overfeeding coupled with limited physical activity (Carter et al., 2009c). Horses evolved to spend many hours every day roaming vast areas of land whilst grazing low-quality forage (Johnson et al., 2010); it is now common for these animals to be confined to stables with only short periods of turn-out and provided with high-energy concentrate feeds, or to have unrestricted access to improved pastures. Individual susceptibility to obesity may also be heightened by the increased metabolic efficiency afforded by certain genetic and epigenetic factors (Speakman, 2004).

1.6.2 Obesity and insulin dysregulation

Obesity is an essential risk factor for the development of cardiovascular disease and type 2 diabetes mellitus in humans with MetS (Fulop et al., 2006; Alberti et al., 2009). The link between obesity and insulin resistance is well established in humans and laboratory animal species (Reaven, 1991; Fellmann et al., 2013). Adipose tissue is no longer dismissed as a quiescent site of lipid storage, and is now recognised as an active endocrine organ in many

\(^{1}\) http://www.who.int/topics/obesity/en/
mammalian species (Radin et al., 2009). Some of the important endocrine functions mediated by adipose-derived hormones include the modulation of insulin sensitivity, vascular function and immune status (Maury and Brichard, 2010). Enlarged adipose depots release increased amounts of non-esterified fatty acids, proinflammatory cytokines and other factors that are implicated in the development of insulin resistance (Khan et al., 2006). Swollen adipocytes experience cellular stress as a result of local hypoxia due to endothelial dysfunction and poor oxygen diffusion (Trayhurn et al., 2014). The inability of swollen adipocytes to take up excess lipid may also contribute to ectopic lipid deposition and lipotoxicity in other tissues of the body (van Herpen and Schrauwen-Hinderling, 2008).

More recently, the long-held view of an obligatory causal association between obesity and insulin dysregulation has been questioned by the description of a ‘metabolically healthy obese’ phenotype (Wildman et al., 2008). It is recognised that a proportion of obese individuals might not be at an increased risk of metabolic complications; however, there is no universally accepted criteria to define this clinical scenario (Stefan et al., 2013). It has been proposed that obesity may only represent a risk to health when an underlying genetic susceptibility to the development of diseases is present, which can be exacerbated by lifestyle and dietary factors (Navarro et al., 2015). This idea supports the definition of obesity proposed by the WHO, in which obesity is more than just a threshold amount of adipose tissue, but a pathophysiological description of dysfunctional adipose depots that are potentially harmful to health.

1.6.3 Equine studies

The significance of equine obesity was highlighted by the inclusion of generalised obesity, or regional adiposity, as a central feature of EMS by the ACVIM consensus panel (Frank et al., 2010). An association between obesity and insulin dysregulation has been demonstrated in numerous cross-sectional studies of equids (Hoffman et al., 2003; Frank et al., 2006; Treiber et al., 2006; Carter et al., 2009c). One potential limitation of these studies is that horses and ponies with insulin dysregulation were examined when they were already obese, making it difficult to separate obesity and insulin dysregulation from one another. To further examine these relationships, Carter et al. (2009b) conducted a controlled study of diet-induced weight gain in a cohort of Arabian geldings. These horses were fed approximately 200% of daily
digestible energy (DE) requirements for 16 weeks, resulting in a 20% increase in BW. Significant insulin dysregulation was observed following the induction of obesity; SI decreased by 70% with a compensatory 400% increase in AIRg. In contrast, other investigators failed to demonstrate significant changes in insulin sensitivity after diet-induced weight gain in Thoroughbreds (Quinn et al., 2008). Differences in the breeds of animal, study diets and final level of adiposity may explain some of these observations.

The association between obesity and insulin dysregulation is not straightforward; it is also recognised that lean animals can be insulin resistant and that obese animals can be insulin sensitive (Johnson, 2002; Geor, 2008). For example, Bailey et al. (2007; 2008) found no differences in body condition between insulin resistant laminitis-prone ponies and insulin sensitive control ponies. One explanation for these inconsistent observations is that obesity can be considered a modifying factor, with the genetics of an individual animal responsible for the degree of insulin dysregulation (Frank and Tadros, 2014). Given that insulin sensitivity can be influenced by dietary factors independent of obesity (Hoffman et al., 2003; Treiber et al., 2005a), further investigation of these relationships is required.

1.7 Assessment of adiposity

Studies of obesity rely on validated techniques that can accurately assess the adiposity of an individual. Various invasive and non-invasive techniques have been described in human and animal studies. However, due to the large size and potentially fractious nature of horses, some of the most accurate techniques used in humans cannot be practically applied to equids. In human studies, hydrodensitometry is considered the ‘gold standard’ technique to assess adiposity (Brodie et al., 1998). Based on Archimedes’ principle that an object will displace its own volume of water, subjects are required to hold their breath whilst remaining completely submerged in water for a short period of time. It is self-evident that hydrostatic weighing is impractical for use in animal studies. Another technique that is commonly used in human research studies is dual energy x-ray absorptiometry (DEXA). This technique uses two low energy x-ray beams to estimate fat-free mass, fat mass and bone density based on perturbations in the passage of x-ray beams through different tissues (Haarbo et al., 1991). Although this technique has found use in veterinary studies of dogs and cats, it cannot be practically applied to horses due to their large size.
1.7.1 Body condition score

The body condition score (BCS) was originally developed for use in livestock species and subsequently adapted to the horse by Henneke et al. (1983). The subjective appraisal of fat cover at specific locations of the body yields a measure of generalised adiposity that accounts for differences in how individual animals deposit subcutaneous fat. Several variations of the BCS have been described in horses. The basic principles of visual inspection and palpation are similar between systems, with the important differences being the number of areas inspected and the range of scores that can be assigned at each location. For example, Carroll and Huntington (1988) described a BCS system that scores three locations of the body (neck, back and ribs, and pelvis) on a scale of 0 to 5 that was been purported to be the most user-friendly due to the small number of measurements required. Despite this claim, the most commonly used BCS systems employed in equine studies remain based on the original 1 to 9 scale of Henneke et al. (1983). The Kohnke (1992) adaptation of the original system is one of the most popular, in which six areas of the body are scored from 1 to 9: neck, shoulder, withers, ribs, loin and tail head (Table 1-2; Figure 1-1). An overall BCS is then calculated as the average of scores from the six locations.

Body condition scoring is the most practical method available to assess adiposity in horses. However, there are important limitations that must be considered. The relationship between BCS and total body fat mass (TBFM) in horses is logarithmic in nature (Dugdale et al., 2012). This is due to the fact that only subcutaneous adiposity is assessed, without an ability to account for intra-abdominal fat. The slope of the regression curve is essentially linear for BCS values of less than 6 out of 9. However, when BCS values are greater than 7 out of 9, a relatively small change in BCS is reflective of a much larger change in TBFM due to the expansion of intra-abdominal fat (Dugdale et al., 2012). Breed-related differences in body conformation and the regional distribution of adiposity may also affect the utility of BCS systems. Adaptations of the Henneke et al. system are most applicable to the light breeds of horse in which they were first described (e.g. Quarter Horses, Thoroughbreds and Standardbreds). Modifications of the Henneke et al. system have been reported for other breeds, such as that described for Warmbloods (Kienzle and Schramme, 2004), although a system for specific use in pony breeds has not yet been developed.

For practical reasons, the vast majority of equine studies assess adiposity using BCS, with an arbitrary cut-off value used to define obesity often set at greater than 7 out of 9 (Dugdale et
al., 2011a). Associations have been shown between obese BCS and evidence of insulin dysregulation (Frank et al., 2006; Carter et al., 2009b), with higher BCS also associated with previous or future episodes of pasture-associated laminitis (Treiber et al., 2006; Carter et al., 2009c).

1.7.2 Cresty neck score

It has been anecdotally reported that horses and ponies with a ‘cresty neck’ are at higher risk of developing pasture-associated laminitis (Johnson, 2002). Subsequent studies have confirmed that excessive neck crest adiposity is associated with insulin dysregulation and laminitis (Frank et al., 2006; Treiber et al., 2006; Carter et al., 2009c). A scoring system to specifically evaluate regional adiposity along the crest of the neck has been described by Carter et al. (2009a). The cresty neck score (CNS) rates the accumulation of fat around the nuchal ligament on a 0 to 5 scale (Figure 1-2). Prior to the advent of the CNS, neck crest adiposity was evaluated using morphometric measurements such as neck circumference, neck circumference-to-height ratio and neck crest thickness (Frank et al., 2006; Bailey et al., 2008). The CNS suffers from the same limitations as BCS in that it provides a subjective assessment of adiposity. However, it provides another practical method for researchers, veterinarians and owners to assess adiposity in horses and ponies.

The rationale for such a practical scoring system is that certain regional adipose depots may have a more profound biological effect than others. In humans, visceral adiposity is more closely associated with cardiovascular disease and type 2 diabetes mellitus than generalised adiposity (Fain et al., 2004). Therefore, waist circumference (an indicator of visceral adiposity) has largely replaced body mass index (an indicator of generalised adiposity) in many of the current diagnostic criteria for MetS (Alberti et al., 2009). The unique biological behaviour of the neck crest adipose depot in horses was proposed by Burns et al. (2010) when gene expression of the proinflammatory cytokines interleukin (IL)-1β and IL-6 were found to be higher in nuchal ligament adipose tissue compared with other sites; a finding that was supported by a subsequent study (Bruynsteen et al., 2013). However, the significance of this finding is not clear and has not been elaborated upon.

The relationship between CNS and pasture-associated laminitis is not straightforward, as demonstrated by Bailey et al. (2007). Laminitis-prone ponies that were examined did not
exhibit a ‘cresty neck’ when compared with non-laminitic control ponies. It is important to note that neck crest adiposity is unlikely to be completely independent of generalised adiposity, although there is some evidence that seasonal fluctuations in CNS and BCS are not perfectly aligned (Giles et al., 2015). In many instances, it is difficult to separate the effect of a ‘cresty neck’ from that of generalised obesity.

1.7.3 Morphometric measurements

A range of different morphometric measurements have been evaluated in equine studies, although none of these have gained wide acceptance or utility. These measurements include chest and abdominal girths, body height and length. Formulas have been used to create a body mass index (BMI) for horses. Donaldson et al. (2004) described one BMI for use in horses as: BW (kg) / [height (m)]^2; while Carter et al. (2009a) described another BMI adapted from small animal species as: BW (kg) / [length (m) x height (m)]. Several various ratios of girth, height and length measurements have also been evaluated (Carter et al., 2009a), although there has been little subsequent use of morphometric measurements in the assessment of adiposity in equine studies.

The measurement of subcutaneous fat depth using ultrasound has been used to estimate fatness in horses (Westervelt et al., 1976; Gentry et al., 2004; Kearns et al., 2006; Carter et al., 2009b). However, this approach has been questioned due to inconsistencies between the anatomical locations described and variations in the breed and physiological status of the animals studied (Dugdale et al., 2011b). For example, the formula used to calculate body fat percentage by Carter et al. (2009b): 6.22 + [5.07 x rump fat thickness (cm)], yielded results that were disparate to body fat % values derived from deuterium oxide dilution calculations in a subsequent study (Carter et al., 2010).

1.7.4 Isotope dilution

The measurement of total body fat mass (TBFM) using isotope dilution has become the ‘gold standard’ technique for assessing adiposity in living horses (Dugdale et al., 2011c). Deuterium (‘heavy hydrogen’; chemical symbol ²H or D) is a stable isotope of hydrogen that contains a neutron within the nucleus. Deuterium oxide (‘heavy water’; chemical symbol
D₂O) is a molecule that behaves similarly to water (H₂O) but can be independently measured using gas isotope ratio mass spectrometry. The principle of isotope dilution is that an administered weight of D₂O will equilibrate within all water-based tissues in the body (and not within lipid-based tissues). Measurement of plasma D₂O concentration after equilibration can be extrapolated to calculate total body water, total body fat-free mass and TBFM.

This technique has been successfully applied to dogs (Son et al., 1998), and validated in equids by comparing D₂O dilution results with TBFM obtained through post mortem anatomical dissection in ponies (Dugdale et al., 2011c). Briefly, blood samples are collected immediately before and 4 hours after a dose of 0.12 g/kg BW D₂O is administered intravenously. Feed and water is withheld during the equilibration period. The syringe containing D₂O is weighed before and after to determine the exact weight administered. Plasma samples are then analysed using gas isotope ratio mass spectrometry to determine D₂O concentrations. The calculations reported by Dugdale et al. (2011c) that are required to arrive at TBFM in equids are included in Appendix 1-1.

1.8 Adipokines

The biologically active molecules that are synthesised and secreted by adipose tissue, called adipokines, have various and wide-ranging effects including the regulation of energy metabolism, cardiovascular and immune functions (Rabe et al., 2008). The important function of adipokines is highlighted in studies of transgenic lipoatrophic mice, which are almost entirely deficient in adipose tissue. These mice exhibit a multitude of metabolic derangements, including severe insulin resistance, which can be completely reversed following exogenous fat transplantation (Reitman and Gavrilova, 2000).

Adipokine dysregulation is an important feature of obesity-related disorders in human patients (Piya et al., 2013). In many cases, it remains unclear whether adipokine dysregulation is a cause or consequence of obesity (Deng and Scherer, 2010). A number of adipokines have been identified, including leptin, adiponectin, apelin, visfatin, omentin and resistin. The two best-characterised adipokines in human and rodent studies are leptin and adiponectin, which are also the adipokines that have received the most attention in equine studies to date (Radin et al., 2009).
1.8.1 Leptin

1.8.1.1 General physiology

Leptin is a peptide hormone that is primarily secreted by mature adipocytes in a constitutive manner (Radin et al., 2009). The main function of leptin is the regulation of energy metabolism, with the release of leptin dependent on energy flux within WAT depots (Jequier, 2002). Leptin exerts its central effects through the satiety centre of the hypothalamus by acting to inhibit feed intake and increase energy expenditure via thermogenesis. Leptin has therefore been referred to as the ‘satiety hormone’ due to its ability to suppress appetite. The classic transgenic mouse model of obesity (the ob/ob mutant) is the result of an absolute leptin deficiency (Houseknecht and Portocarrero, 1998). These mice gain weight rapidly because their inability to detect satiety results in the excessive intake of feed. In addition to the regulation of energy metabolism, leptin has other important physiological actions including the modulation of insulin sensitivity and the regulation of reproductive and immune functions (Margetic et al., 2002).

Serum leptin concentrations are closely related to body mass. However, some of the variability observed between human patients is independent of adiposity. It has also been observed that the downstream effects of leptin signalling (reduced food intake and increased energy expenditure) are not always appropriate for a given degree of hyperleptinaemia. This has led researchers to hypothesise that a state of leptin resistance may perpetuate obesity in some individuals (Jequier, 2002). The development and clinical application of potential ‘leptin sensitisers’ for the treatment of MetS is a growing field within human therapeutics (Mantzoros et al., 2011).

1.8.1.2 Equine studies

Leptin is the most extensively investigated adipokine in equine studies, with hyperleptinaemia mentioned by the ACVIM consensus panel as a component of EMS that warrants additional consideration (Frank et al., 2010). Similar to human and rodent studies, serum leptin concentrations are positively correlated with adiposity in horses (Buff et al., 2002; Gentry et al., 2002; Kearns et al., 2006; Pratt-Phillips et al., 2010). In studies that considered an association with insulin dysregulation, hyperleptinaemia was detected in obese horses that were also insulin resistant or hyperinsulinaemic (Frank et al., 2006; Carter et al.,
Additionally, Carter et al. (2009c) identified hyperleptinaemia (>7.3 ng/mL) as one of the risk factors, along with hyperinsulinaemia (>32 mIU/L) that could be used to predict the development of laminitis in a cohort of ponies. Obesity and regional adiposity were also included in these risk factors, so it is unclear if leptin was independent of, or simply reflective of, fat mass.

Whether a state of leptin resistance occurs in horses is not clear. One previous study showed that variations in leptin concentrations existed between horses of similar body condition (Cartmill et al., 2003). These horses also had evidence of insulin dysregulation, leading the investigators to hypothesise that leptin resistance may occur in equids. However, other studies have not supported this hypothesis, with leptin concentrations appearing to reflect adiposity in a quantitative rather than qualitative manner (Frank et al., 2006; Carter et al., 2009c; Pleasant et al., 2013). There is some evidence that leptin gene expression is increased in nuchal adipose tissue, suggesting that this depot might make an important contribution to overall leptin levels (Bruynsteen et al., 2013).

### 1.8.2 Adiponectin

#### 1.8.2.1 General physiology

Adiponectin is a peptide hormone that is exclusively release from mature adipocytes, which in contrast to leptin is typically inversely proportional to adiposity (Maury and Brichard, 2010). Adiponectin circulates in three recognised complexes: 90-kDa trimers, 180-kDa low-molecular weight hexamers and >360-kDa high-molecular weight (HMW) multimers, with the HMW form recognised to be the most biologically active (Schraw et al., 2007). Hypoadiponectinaemia has been postulated to play a role in the pathogenesis of several comorbidities in humans with MetS, due to a reduction in the recognised anti-inflammatory, anti-arthrogenic and insulin-sensitising actions of adiponectin (Brochu-Gaudreau et al., 2010; Fisman and Tenenbaum, 2014).

In humans, the degree of hypoadiponectinaemia is closely linked to the severity of insulin resistance, with one theory being that the production of proinflammatory cytokines within enlarged adipose depots acting as paracrine inhibitors of adiponectin expression (Hivert et al., 2008). The administration of exogenous adiponectin has been shown to reverse insulin
resistance in laboratory animal models of type 2 diabetes mellitus (Yamauchi et al., 2001). There is also evidence that people with the ‘metabolically healthy obese’ phenotype may have higher adiponectin levels than obese people with MetS (Aguilar-Salinas et al., 2008). The principle receptors for adiponectin are found within the liver and skeletal muscle (Kadowaki and Yamauchi, 2005). Adiponectin improves insulin sensitivity by increasing glucose disposal through the upregulation of GLUT4 glycoproteins; it also increases the utilisation of fatty acids for energy via β-oxidation and decreases hepatic gluconeogenesis (Whitehead et al., 2006).

1.8.2.2 Equine studies

Similar to human and rodent studies, adiponectin concentrations are negatively correlated with basal insulin concentrations and inversely proportional to fat mass in horses (Kearns et al., 2006; Wooldridge et al., 2012). A commercial ELISA has been validated for use in the horse, making the investigation of this adipokine in equine studies more achievable (Wooldridge et al., 2012). When Wray et al. (2013) considered laminitis status, previously-laminitic ponies had lower adiponectin concentrations than control ponies. A short period of low intensity exercise did not show any beneficial effect on plasma adiponectin concentrations (Menzies-Gow et al., 2014). It is not clear whether hypoadiponectinaemia is a cause or a consequence of insulin dysregulation, nor is it clear whether low adiponectin levels are associated with the pathophysiology of endocrinopathic laminitis. Due to the links between adiponectin and both insulin dysregulation and laminitis status, further investigation of this adipokine in equine studies is warranted.

1.9 Obesity-associated inflammation

1.9.1 General physiology

In humans, obesity is regarded a state of chronic low-grade inflammation, with cytokine production found to be an important element of adipose tissue dysfunction in patients with MetS (Lumeng and Saltiel, 2011). Whether this is the cases in horses has not been fully determined, but obesity-associated inflammation was identified by the ACVIM consensus panel as a potential component of EMS that warrants further consideration (Frank et al.,
The expansion of adipose depots can lead to a proinflammatory state through the development of oxidative stress, endothelial cell damage and phenotypic shifts in resident macrophages (Maury and Brichard, 2010). Adipokine dysregulation and inflammation often go hand-in-hand, although it is unclear whether alterations in adipokines are a cause or consequence of inflammation (Trayhurn and Wood, 2004).

Dysfunctional adipose tissue produces a range of potentially deleterious cytokines including TNF-α, IL-6, plasminogen activator-inhibitor-1 (PAI-1) and monocyte chemoattractant protein-1 (MCP-1) (Shoelson et al., 2006). The increased recruitment of monocytes into adipose depots by PAI-1 and MCP-1 will potentiate the release of cytokines in a self-perpetuating fashion. It is proposed that systemic inflammation can interfere with insulin signalling, leading to insulin resistance and hyperinsulinaemia (Kern et al., 2001; Piya et al., 2013). Another clinically-important consequence of systemic inflammation is vascular endothelial dysfunction (Dooley et al., 2014). Chronic low-grade inflammation may therefore represent at least part of the link between type 2 diabetes mellitus and cardiovascular disease as the clinical manifestations of MetS in people (Maury and Brichard, 2010).

1.9.2 Equine studies

There is conflicting evidence about whether obesity represents a proinflammatory state in the horse (Frank and Tadros, 2014). Early studies examining the relationship between obesity and inflammation reported positive correlations between BCS and the proinflammatory cytokines IL-1 and TNF-α (Vick et al., 2007; Carter et al., 2009). However, age may have been an important confounder in these studies due to so-called ‘inflamm-aging’, the process by which advancing age potentiates the inflammatory response (Adams et al., 2009). Subsequent studies of equids have failed to show an association between obesity and cytokine-mediated inflammation or evidence of oxidative stress (Treiber et al., 2009; Holbrook et al., 2012). Suagee et al. (2013) did not find an association between BCS and plasma concentrations of IL-1β, IL-6 and TNF-α, but did detect higher serum amyloid A (SAA) levels in obese horses. Serum amyloid A is an acute phase protein released from the liver and could possibly be a better marker of obesity-associated inflammation in horses (Suagee et al., 2013).
As mentioned previously, differences in the gene expression of IL-1β and IL-6 between different adipose depots have been demonstrated, with higher levels found in the nuchal ligament compared with omental or subcutaneous sites (Burns et al., 2010; Bruynsteen et al., 2013). The potential significance of this finding is unclear, since no overall differences in the gene expression of TNF-α, IL-1β, IL-6 and MCP-1 were detected between insulin sensitive and insulin resistant horses (Burns et al., 2010). The expression of IL-1β, IL-6 and TNF-α has also been measured in the digital lamellae of ponies and was found to be similar between lean and obese ponies (Burns et al., 2015). When laminitis status has been considered, one study found that ponies with a history of recurrent laminitis had significantly higher plasma concentrations of TNF-α compared with normal ponies (Treiber et al., 2009); whilst another study found no differences between TNF-α or SAA concentrations between normal ponies and previously-laminitic ponies (Wray et al., 2013).

The current lack of evidence associating the EMS phenotype with a proinflammatory state may indicate that horses and ponies adapted to store adipose tissue without the development of potentially deleterious inflammation. However, further investigation of the associations between obesity, insulin dysregulation and inflammation are required to fully elucidate the mechanisms that might link one to another. The induction of a proinflammatory state through the infusion of lipopolysaccharide is known to decrease insulin sensitivity in healthy horses (Toth et al., 2008); changes that are more pronounced in horses with EMS (Tadros et al., 2013). There is also evidence that hyperinsulinaemia per se can induce low-grade inflammation, as determined by the measurement of TNF-α and IL-6 concentrations in horses subjected to an 8-hour EHC procedure (Suagee et al., 2011).

1.10 Diet

1.10.1 Dietary carbohydrates

The high prevalence of MetS in recent years has been associated with increased consumption of dietary carbohydrates, particularly fructose (Basciano et al., 2005). It has been suggested that it is the dietary aggravation of an underlying genetic predisposition that results in the clinical expression of MetS in people (Reaven, 2005). Just as MetS has been considered a ‘lifestyle disease’, there are interesting parallels that have been drawn to a role for diet in the
emergence of EMS in horses and ponies (Johnson et al., 2013). Equids evolved to live on relatively sparse pasture environments, particularly over winter when they would utilise their fat reserves. The high levels of sugars that are available to horses in improved pastures and high energy concentrate feeds may induce metabolic changes that become detrimental to health (Harris et al., 2006).

Diet is an essential consideration in the investigation of how obesity and insulin dysregulation are expressed in equids. The dietary glycaemic load will influence the degree of hyperinsulinaemia experienced by an animal, whilst the differential between caloric intake and energy expenditure will have direct bearing on the accumulation of adiposity. Studies have shown that the adaptation to high glycaemic ‘sweet feed’ meals is capable of inducing compensated insulin resistance in weanlings and mature horses (Hoffman et al., 2003; Treiber et al., 2005a). The chronic adaptation to high glycaemic diets, causing repeated episodes of hyperinsulinaemia, can result in the down-regulation of insulin receptors in target tissues. This will lead to a self-perpetuating cycle of insulin dysregulation that can be modified by factors including diet, genetic predisposition and the presence of obesity (Figure 1-3; adapted from Frank and Tadros, 2014).

Individual or breed susceptibility to hyperinsulinaemia may play a role in the development of diet-induced insulin dysregulation. As mentioned earlier, Carter et al. (2009b) provided Arabian geldings with multiple daily ‘sweet feed’ meals over a 16-week period, inducing obesity alongside marked insulin resistance. Quinn et al. (2008) provided Thoroughbred geldings with a similar diet, but was unable to demonstrate significant changes in insulin sensitivity over 28 weeks. It is difficult to draw definitive conclusions from these studies, as neither enrolled a control group for comparison. A previous study demonstrated differences in the glycaemic and insulinaemic responses of ponies and Standardbred horses to oral glucose (Jeffcott et al., 1986). Therefore, one hypothesis for these observations is that the Arabians experienced greater postprandial insulin responses than the Thoroughbreds. The provision of excessive dietary NSC to an individual predisposed to hyperinsulinaemia may represent the risk of developing laminitis (Harris et al., 2006).

The carbohydrate and protein composition of the diet is also important to consider. Plant carbohydrates can be divided into structural carbohydrates, the fibrous cell wall component including cellulose, hemicellulose and lignin; and non-structural carbohydrates (NSC), the cell contents including simple sugars (mono- and disaccharides), oligosaccharides (including
fructans), fructan polysaccharides and starch (Harris and Geor, 2009). The equine digestive tract lacks the enzymes capable of hydrolysing cellulose or hemicellulose, which are instead digested via bacterial fermentation in the hindgut. The NSC fraction is of paramount importance, as starch and simple sugars are readily digested and rapidly absorbed from the small intestine, prompting postprandial glycaemic and insulinaemic responses. Fructan carbohydrates (the reserve carbohydrate of temperate grasses) have long been regarded as indigestible by mammalian species due to the lack of appropriate enzymes, but there is some recent evidence that fructans may undergo a degree of digestion in the foregut of horses (Coenen et al., 2006; Ince et al., 2014). Multiple factors, including the plant cultivar, climatic conditions and stage of lifecycle will affect the relative amounts of NSC within pasture (Longland and Byrd, 2006). Protein content may also play an influential role, as certain amino acids such as arginine can exert powerful insulinogenic effects (Clemmensen et al., 2013).

1.10.2 Glycaemic index

The glycaemic index (GI) has been used in human dietetics to indicate the relative effect that a particular food has on postprandial blood glucose levels (Jenkins et al., 1981). Although the GI has been somewhat contentious due to variations in methodology (Wolever et al., 1991), the adoption of tighter international standards may have improved its utility in more recent times (Brand-Miller and Buyken, 2012). The glycaemic and insulinaemic responses to various feeds have been evaluated in a multitude of equine studies (Williams et al., 2001; Jose-Cunilleras et al., 2004; Rodiek and Stull, 2007; Vervuert et al., 2009; Nielsen et al., 2010). Despite these data, the application of a generic GI in equine dietetics remains fraught due to the enormous variability in glucose and insulin responses observed in some studies (Kronfeld et al., 2004a; Harris and Geor, 2009). Additionally, the examination of glucose responses only does not acknowledge the individual differences in insulin responses for a given degree of hyperglycaemia.
1.10.3 Pasture consumption

The concept of pasture-associated laminitis implicitly acknowledges a role for the consumption of NSC-rich forage in the development of laminitis (Geor, 2010). The peak incidence of laminitis occurs during the spring and summer months, when pasture NSC content is at its highest (Polzer and Slater, 1996; Menzies-Gow et al., 2010a). As mentioned previously, the provision of an intragastric bolus of oligofructose to horses is one of the experimental models of laminitis induction, suggesting that dietary hindgut overload of this form of carbohydrate may be a means by which pasture consumption can induce laminitis (van Eps and Pollitt, 2006). However, it has been questioned whether the amount of oligofructose administered during the induction of laminitis (10 g/kg BW) is reflective of voluntary pasture consumption (Harris et al., 2006). Additionally, the complex nature of temperate grass fructans is different from the type of fructans used in experimental models of laminitis (Longland and Byrd, 2006). In light of the more recently described models of hyperinsulinaemic laminitis (Asplin et al., 2007; de Laat et al., 2010b), it is widely accepted that pasture-associated laminitis is more likely to be associated with the aggravation of insulin dysregulation and hyperinsulinaemia by pasture intake (Frank and Tadros, 2014).

Certain individuals are predisposed to recurrent episodes of pasture-associated laminitis whilst others continue to graze pasture with seeming impunity. Bailey et al. (2008) found that the expression of certain phenotypic traits of associated with EMS changed with season in laminitis-prone ponies kept at pasture. Insulin resistance, hypertriglyceridaemia and hypertension were present during summer but not winter, indicating that summer pastures may induce a metabolic shift in these animals that could be associated with the development of laminitis. Additionally, exaggerated insulin responses were detected when a group of previously-laminitic ponies were fed inulin (a form of fructan carbohydrate) under controlled conditions (Bailey et al., 2007). Hyperinsulinaemia was also a consistent finding in previously-laminitic ponies when fed different types of NSC, including glucose, fructose and inulin, at different times of the year (Borer et al., 2012b).

1.10.4 Dietary fats

The replacement of sugar and starch with fat and fibre has been advocated for the management of horses and ponies with insulin dysregulation (Kronfeld et al., 2005b).
Postprandial glucose and insulin responses are reduced when NSC is substituted with fat and fibre (Williams et al., 2001; Zeyner et al., 2006). Although the equine gastrointestinal system has evolved to utilise feedstuffs that are low in fat, the digestion of relatively large amounts of fat (up to 25% of DE intake) is well tolerated (Harris et al., 1999; Kronfeld et al., 2004b). In comparison, high fat diets are commonly used to induce insulin resistance in laboratory animal models of human type 2 diabetes mellitus (Winzell and Ahren, 2004). These diets are often much higher in fat content (over 50% daily DE) and contain predominantly saturated fatty acids, which are likely to induce insulin resistance through the process of lipotoxicity.

Diets containing supplementary fat have been used in horses to verify that the reduced insulin sensitivity encountered during adaptation to ‘sweet feed’ meals is due to the sugar and starch content, rather than the energy content per se (Hoffman et al., 2003; Treiber et al., 2005a). Similarly, the use of an isocaloric fat-fortified ration enabled investigators to separate the effect of diet-induced obesity from the provision of high-NSC meals (Quinn et al., 2008). A methodological evaluation of dietary fat supplementation in horses with insulin dysregulation has not been performed. It is likely that the avoidance of sugar and starch is of most benefit, rather than an insulin-sensitising effect of dietary fat. In obese animals, supplementary fat is rarely required to provide additional calories; however, a subset of horses and ponies with insulin dysregulation, such as those with PPID, may require additional energy to maintain weight when sugar and starch is ideally avoided.

1.11 Incretins

The insulin response to glucose is potentiated when a glycaemic load is administered orally, as opposed to intravenously (Hampton et al., 1986). This observation is due to the incretin effect. Incretins are hormones that are released from specialised enteroendocrine cells within the intestinal wall in response to the ingestion of sugars, amino acids and fats (Baggio and Drucker, 2007). The principle action of incretins is to minimise postprandial hyperglycaemia by enhancing insulin secretion and delaying gastric emptying; in addition, incretins also enhance pancreatic β cell mass (De Graaf-Roelfsema, 2014). High incretin concentrations are associated with insulin resistance and other components of MetS in humans (O’Neill and O’Driscoll, 2015).
Two incretin hormones have been characterised in people and animals: glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP; previously called gastric inhibitory peptide), which are released from L cells and K cells, respectively (Holst and Gromada, 2004). The actions of both GLP-1 and GIP are regulated by the rapid degradation of these incretins by dipeptidyl peptidase-4 (DPP-4); therefore, insulin secretion can be intensified by an increase in incretin release or a reduction in incretin degradation by DPP-4 (Baggio and Drucker, 2007). In human medicine, both incretin analogues and DPP-4 inhibitors have been evaluated for use in patients with type 2 diabetes mellitus and pancreatic β-cell insufficiency (Drucker and Nauck, 2006).

A functional enteroinsular axis in equids has been confirmed through the measurement of both GIP (Duhlmeier et al., 2001) and GLP-1 (Chameroy et al., 2011); however, there is still much to be learned about the enteroinsular axis of horses (De Graaf-Roelfsema, 2014). Duhlmeier et al. (2001) found higher glucose-to-insulin ratios during an OGTT, which were associated with an increase in circulating GIP, when compared with an equivalent intravenous glucose tolerance test. Shetland ponies were found to have greater glucose and insulin concentrations than large-breed horses; however, glucose-to-insulin ratios were similar, suggesting a comparable enteroinsular axis between these breeds. It has been proposed that chronic incretin stimulation may contribute to β cell hyperplasia and hyperinsulinaemia in horses and ponies with EMS; incretins may also help to explain why certain animals demonstrate postprandial hyperinsulinaemia despite normal insulin sensitivity (Frank and Tadros, 2014).
1.12 Aims and objectives

The relationships between diet, obesity and insulin dysregulation in equids require further investigation due to their association with laminitis. It remains unclear whether periods of diet-induced hyperinsulinaemia, the presence of obesity, or a combination of both are responsible for the development of insulin dysregulation in horses and ponies. A better understanding of how genetic and environmental factors contribute to the development of the EMS phenotype is crucial to improving the management of animals at risk of laminitis.

To examine the metabolic pathways that may underlie the relative predisposition of different breeds to the EMS phenotype, the studies in this thesis selected three distinct breed groups for comparison: Standardbreds, an example of a horse breed that does not appear predisposed to obesity, insulin dysregulation or laminitis; Andalusians, a Spanish horse breed that commonly exhibits a tendency toward obesity (‘easy keeper’) and predisposition to laminitis (Riber et al., 1995); and ponies, which are considered the primordial equine breed predisposed to insulin dysregulation and laminitis.

The primary aim of the studies presented in this thesis was to determine whether periods of increased blood glucose and insulin are the major factors causing insulin dysregulation in horses and ponies, or whether weight gain alone could promote the appearance of the insulin resistant phenotype. The primary hypothesis was that a high glycaemic diet would be a more important influence on the development of insulin dysregulation than the development of obesity per se.

Specific research questions included:

1. Are there innate differences in glucose and insulin dynamics between different equine breeds, independent of the effects of obesity or a high glycaemic diet?
2. Do animals that gain weight on a high glycaemic diet develop more severe insulin resistance than animals that consume an isocaloric low glycaemic diet?
3. Are there differences between breeds in the metabolic changes that occur during weight gain or adaptation to a high glycaemic diet?
4. Do circulating levels of adipokines, such as leptin and adiponectin, or proinflammatory cytokines change during weight gain in horses and ponies?
5. Do incretins play a role in the postprandial insulin responses of horses and ponies adapted to high glycaemic meals?
**Table 1-1:** Purported similarities between human metabolic syndrome (MetS) and equine metabolic syndrome (EMS).

<table>
<thead>
<tr>
<th></th>
<th>MetS</th>
<th>EMS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin</strong></td>
<td>Hyperinsulinaemia, insulin resistance</td>
<td>Hyperinsulinaemia, insulin resistance</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>Fasting hyperglycaemia</td>
<td>Normoglycaemia</td>
</tr>
<tr>
<td><strong>Obesity</strong></td>
<td>Increased body mass index</td>
<td>Increased body condition score</td>
</tr>
<tr>
<td><strong>Regional adiposity</strong></td>
<td>Increased waist circumference</td>
<td>Increased neck circumference, increased cresty neck score</td>
</tr>
<tr>
<td><strong>Dyslipidaemia</strong></td>
<td>Increased triglycerides, reduced HDL-C</td>
<td>Increased triglycerides, increased NEFA, increased HDL-C</td>
</tr>
<tr>
<td><strong>Blood pressure</strong></td>
<td>Hypertension</td>
<td>Hypertension</td>
</tr>
<tr>
<td><strong>Adipokines</strong></td>
<td>Hyperleptinaemia, hypoadiponectinaemia</td>
<td>Hyperleptinaemia, hypoadiponectinaemia</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td>Increased TNF-α, IL-1β, IL-6</td>
<td>Conflicting evidence for proinflammatory state</td>
</tr>
</tbody>
</table>

Abbreviations: HDL-C, high-density lipoprotein-cholesterol; IL, interleukin; NEFA, non-esterified fatty acids; TNF, tumour necrosis factor. References: Frank et al., 2006; Kearns et al., 2006; Treiber et al., 2006; Bailey et al., 2008; Alberti et al., 2009; Carter et al., 2009c; Holbrook et al., 2012; Wooldridge et al., 2012; Piya et al., 2013. This table is the original work of the author that preceded the publication of a similar table by McCue et al. (2015).
**Table 1-2:** Body condition score (BCS) chart described by Kohnke (1992) as a modification of Henneke et al. (1983).

<table>
<thead>
<tr>
<th>BCS</th>
<th>General condition</th>
<th>Neck</th>
<th>Shoulder</th>
<th>Withers</th>
<th>Ribs</th>
<th>Loin</th>
<th>Tail head</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Very poor</td>
<td>Individual bone structure visible; feels bony</td>
<td>Bone structure very visible and sharp to touch</td>
<td>Bones easily visible; no fat; razor-like</td>
<td>Ribs very visible and skin furrows between ribs</td>
<td>Spine bones visible; ends feel pointed</td>
<td>Tail head and hips very visible</td>
</tr>
<tr>
<td>2</td>
<td>Very thin</td>
<td>Bones just visible; animal emaciated</td>
<td>Bone structure can be outlined</td>
<td>Withers obvious; very minimal fat covering</td>
<td>Ribs prominent; slight depression between ribs</td>
<td>Slight fat covering over spine projections; ends feel rounded</td>
<td>Tail head and hipbones obvious to the eye</td>
</tr>
<tr>
<td>3</td>
<td>Thin</td>
<td>Thin, flat muscle covering; no raised muscle or fat</td>
<td>Shoulder accentuated; some fat cover but thinner than desirable</td>
<td>Withers thin and accentuated with some, although little, fat cover</td>
<td>Slight fat cover over ribs; rib outline obvious to the eye</td>
<td>Fat build-up halfway on vertical spines, but easily visible; spinal bones not felt</td>
<td>Tail head prominent; hip bones appear rounded, but easily visible; pin bones covered</td>
</tr>
<tr>
<td>4</td>
<td>Moderately thin</td>
<td>Some fat; not obviously thin</td>
<td>Shoulder not obviously thin, some fat cover</td>
<td>Withers not obviously thin, edges smooth but prominent</td>
<td>Faint outline visible to the eye</td>
<td>Slight outward ridge along back</td>
<td>Fat can be felt</td>
</tr>
<tr>
<td>5</td>
<td>Moderate</td>
<td>Neck blends smoothly into body, some fat cover</td>
<td>Shoulder blends smoothly into body</td>
<td>Withers smoothly rounded over top</td>
<td>Ribs cannot be seen but can be felt easily</td>
<td>Back level</td>
<td>Fat around tail head beginning to feel spongy</td>
</tr>
<tr>
<td>6</td>
<td>Moderately fleshy</td>
<td>Fat can easily be felt</td>
<td>Fat layer can be felt</td>
<td>Fat can be felt</td>
<td>Fat over ribs feels spongy</td>
<td>May have slight inward crease down back</td>
<td>Fat around tail head feels soft and palpable</td>
</tr>
<tr>
<td>7</td>
<td>Fleshy</td>
<td>Visible fat deposits or lumps along neck</td>
<td>Fat build-up behind shoulder</td>
<td>Fat covering withers is firm</td>
<td>Individual ribs can still be felt</td>
<td>May have slight inward crease down back</td>
<td>Fat around tail head is soft and rounded off</td>
</tr>
<tr>
<td>8</td>
<td>Fat</td>
<td>Noticeable thickening of neck</td>
<td>Area behind shoulder filled in flush with body</td>
<td>Area along withers filled with fat</td>
<td>Difficult to feel ribs</td>
<td>Crease down back evident</td>
<td>Tail head fat very soft and flabby</td>
</tr>
<tr>
<td>9</td>
<td>Extremely fat</td>
<td>Bulging fat</td>
<td>Bulging fat</td>
<td>Bulging fat</td>
<td>Patchy fat over ribs</td>
<td>Obvious deep crease down back</td>
<td>Building fat around tail head</td>
</tr>
</tbody>
</table>

Individual scores are added together and divided by 6 (the number of areas examined) to give the overall condition score. The score is then aligned to the general description.
Figure 1-1: Six areas of the body assessed using the body condition score (BCS) system described by Henneke et al. (1983) and adapted by Kohnke (1992).

Figure 1-2: Cresty neck scoring system (from Carter et al., 2009a).
Figure 1-3: Relationships between hyperinsulinaemia and insulin resistance, with the modifying effects of breed, diet and obesity (adapted from Frank and Tadros, 2014).
Chapter 2 Breed differences in insulin sensitivity and insulinaemic responses to oral glucose in horses and ponies of moderate body condition score

This chapter is presented as the author-accepted manuscript of a peer-reviewed article published in *Domestic Animal Endocrinology* (2014; 47:101–107).

2.1 Overview

This study compared the glucose and insulin dynamics of different equine breeds whilst animals were in moderate body condition and maintained on a forage-only diet (i.e. independent of the potential modifying effects of obesity or a high glycaemic diet).

The central aims of this study were:

- To determine the indices of insulin sensitivity in different breeds using a FSIGT.
- To examine the postprandial glucose and insulin responses of different breeds to a meal containing a large glycaemic load.
- To correlate the indices of insulin sensitivity obtained from the FSIGT with the glucose and insulin responses measured following the study meal.
2.2 Abstract
There may be breed-related differences in the innate insulin sensitivity (SI) of horses and ponies, an important factor believed to be associated with the risk of laminitis. The aim of this study was to measure the glucose and insulin responses of different breeds of horses and ponies in moderate body condition to a glucose-containing meal, and to compare these responses with the indices of SI as determined by a frequently-sampled intravenous glucose tolerance test (FSIGT). Eight Standardbred horses, eight mixed-breed ponies and seven Andalusian-cross horses with a mean (± SEM) body condition score 5.0 (± 0.3) out of 9 were used in this study. Each animal underwent an oral glucose tolerance test (OGTT) in which they were fed a fibre-based ration (2.0 g/kg BW) containing 1.5 g/kg BW added glucose, as well as a standard FSIGT with minimal model analysis.

The glucose response variables from the OGTT were similar between groups; however, the peak insulin concentration was higher in ponies (94.1 ± 29.1 µIU/mL; \(P = 0.003\)) and Andalusians (85.3 ± 18.6; \(P = 0.004\)) when compared with Standardbreds (21.2 ± 3.5). The insulin area under the curve was also higher in ponies (13.5 ± 3.6 IU·min·L\(^{-1}\); \(P = 0.009\)) and Andalusians (15.0 ± 2.7; \(P = 0.004\)) when compared with Standardbreds (3.1 ± 0.6). Insulin sensitivity, as determined by the FSIGT, was lower in Andalusians (0.99 ± 0.18 x 10\(^{-4}\)/[mIU·min]) compared with Standardbreds (5.43 ± 0.94; \(P < 0.001\)), and in ponies (2.12 ± 0.44; \(P = 0.003\)) compared with Standardbreds. Peak insulin concentrations from the OGTT were negatively correlated with SI (\(P < 0.001\); \(r_s = -0.75\)). These results indicate that there are clear breed-related differences in the insulin responses of horses and ponies to oral and intravenous glucose. All animals were in moderate body condition, indicating that breed-related differences in insulin dynamics occurred independent of obesity.

2.3 Introduction
The term equine metabolic syndrome (EMS) has been used to describe the phenotypic characteristics of animals that are at higher risk of developing laminitis [1]. The central components of EMS have been considered to be increased adiposity (generalised obesity or regional adiposity), hyperinsulinaemia and insulin resistance (IR) [1]. As yet, it has not been established whether obesity is a cause or a consequence of IR in horses, nor whether it is essential to the EMS phenotype. Obesity has been proposed to contribute to IR through the
disruption of insulin-signalling pathways by the adipokines and pro-inflammatory cytokines produced by excessive amounts of adipose tissue [2]. However, IR has developed in horses independent of obesity – such as when they become adapted to diets containing large amounts of non-structural carbohydrates (NSC) [3,4]. This is presumably as a result of prolonged periods of post-prandial hyperinsulinaemia causing the down-regulation of insulin receptors in target tissues. To date, it has been difficult to independently assess the role of obesity and hyperinsulinaemia in the development of laminitis in horses with EMS.

A genetic basis for the EMS phenotype has been proposed [5]. It is recognised that ponies and certain breeds of horse (including Morgans, Paso Finos and Quarter Horses) are more commonly affected [6,7]. Andalusian horses (a Spanish warmblood breed) are an example of a breed predisposed to the EMS phenotype, which commonly exhibit a tendency toward obesity (‘easy keeper’) and are considered to be predisposed to laminitis [8]. On the other hand, Standardbred horses do not appear to be predisposed to obesity, IR or laminitis. Previous studies have found oral glucose tolerance to be impaired in obese and laminitis-prone ponies when compared with Standardbred horses, supporting the observation that there may be innate differences between breeds [9,10]. A better understanding of how genetic and environmental factors contribute to the development of EMS is crucial to improving the management of horses and ponies at risk of developing laminitis [11].

The assessment of insulin sensitivity (SI) using a quantitative method such as the insulin-modified frequently-sampled intravenous glucose tolerance test (FSIGT) has been successfully used in horses, although its utility is generally confined to the research setting [12]. Investigators have tried to determine whether the insulinaemic response of horses to a simple oral sugar test can be used as an index of IR [13,14]. In addition to absorbed glucose, other factors such as incretin hormones may contribute to pancreatic stimulation when carbohydrates are given by the oral route [15]. Therefore, oral glucose tolerance test (OGTT) insulin responses may not necessarily with FSIGT insulin responses.

The aim of this study was to examine the glucose and insulin responses of Standardbred horses, ponies and Andalusian horses in moderate body condition to an OGTT and FSIGT. We hypothesised that ponies would have lower SI than horses, which would be reflected by increased insulinaemic responses to a glucose-containing meal.
2.4 Materials and methods

2.4.1 Animals

Eight Standardbred horses (median age 8.0 yr; range 4 to 20 yr), eight mixed-breed ponies (8.5 yr; 5 to 16 yr), and seven Andalusian-cross horses (8.0 yr; 3 to 13 yr) were used in this study. Adiposity was assessed by a single experienced observer, with body condition score (BCS) assessed on a 9-point scale [16,17], and cresty neck score (CNS) assessed using the 5-point scale described by Carter et al. [18]. Mean (± SEM) BCS and CNS were determined to be 4.9 ± 0.3 and 1.5 ± 0.2 for Standardbreds, 5.0 ± 0.2 and 1.6 ± 0.2 for ponies, and 5.3 ± 0.2 and 2.3 ± 0.2 for Andalusians, respectively. Animals were only enrolled in the study if they had no history of laminitis and were deemed to be clinically healthy by a veterinarian, including the exclusion of pituitary pars intermedia dysfunction by a low-dose dexamethasone suppression test [19]. All horses and ponies underwent routine anthelmintic treatments, farriery and dental treatments as appropriate prior to entering large dirt paddocks with ad libitum access to long-stem grass hay (92% dry matter [DM], 7.4 MJ/kg DM, 9.1% NSC). Dry matter hay intake was monitored and estimated to be 2.0% BW for all breed groups throughout the study. Animals were acclimatised to husbandry practices for at least 4 wk prior to any procedures being initiated. The use of animals in this study was approved by the University of Melbourne Animal Ethics Committee (ID 1011918).

2.4.2 Oral glucose tolerance test

Procedures were performed during (Southern Hemisphere) winter, with OGTT completed over a 2 wk period. Individuals were blocked by breed and randomly assigned to the order in which they were tested, ensuring an even distribution of breed groups across the testing period. Horses were allowed continual access to hay and water in the dirt paddocks overnight prior to the test. On the morning of the OGTT, horses were moved to individual yards at 8:00 AM and a jugular catheter was placed to facilitate blood sampling. After collection of a baseline blood sample (10 mL) at 10:00 AM, horses were fed a meal containing 1.5 g/kg BW glucose (Dextrose monohydrate, Sykes, Australia) mixed with soaked soyahull pellets (1.0 g/kg BW; Maxisoy, Energreen Nutrition, Australia) and oaten chaff (1.0 g/kg BW). The amount of glucose was chosen after pilot studies determined this to be the maximum amount
of added glucose reliably consumed in a meal. Further blood samples (10 mL) were collected 15, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min after the meal was provided. Samples were transferred into tubes containing lithium heparin anticoagulant (BD Vacutainer, Plymouth, UK) and placed on ice until centrifugation.

2.4.3 Frequently-sampled intravenous glucose tolerance test

Each animal underwent a FSIGT 3 wk following the OGTT (in the same order as they underwent the OGTT), as previously described [3]. Briefly, an indwelling catheter was placed in the left jugular vein and baseline samples (10 mL) were collected 60 min, 45 min and immediately prior to a glucose bolus of 300 mg/kg BW (40% w/v dextrose solution) administered within 2 min. Twenty min later, an insulin bolus of 20 mIU/kg BW (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was administered into the right jugular vein. Blood samples (10 mL) were collected at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min following the glucose bolus. Samples were transferred to tubes containing lithium heparin and placed on ice until centrifugation.

2.4.4 Plasma assays

Plasma was harvested within 60 min, following centrifugation at 1000 X g for 10 min at 4°C, and 1 mL aliquots were stored at –80°C until analysis. Plasma glucose and insulin concentrations were measured in each sample from the OGTT and FSIGT. Additionally, plasma cortisol was measured in the 0 min sample from the FSIGT for each animal. Glucose concentrations were measured using a hexokinase colourimetric assay (Cayman Chemical Co., Ann Arbor, MI). Insulin and cortisol concentrations were measured using RIA (Coat-A-Count, Siemens Diagnostics, Los Angeles, CA) previously validated for equine samples [20,21]. Intra-assay CV were 0.7 and 3.6 % and inter-assay CV were 0.8 and 5.8 % for glucose and insulin, respectively.
2.4.5 Data analysis

The minimal model of glucose and insulin dynamics [22] was used to interpret the curves generated by the FSIGT, using MinMod Millennium software (version 6.02, University of Pennsylvania, Kennett Square, PA) [3]. Values were obtained for insulin sensitivity (SI), acute insulin response to glucose (AIRg), glucose effectiveness (Sg) and disposition index (DI).

Data analysis was performed using GraphPad Prism software (version 6.02; La Jolla, CA). The Shapiro-Wilk test was used to evaluate data for normality using residual values. Groups were compared using a one-way ANOVA with Tukey’s post hoc test or a Kruskal-Wallis test with Dunn’s post hoc test, as appropriate. For the OGTT, peak concentrations were defined as the highest concentration measured for each animal, and areas under the curve (AUC) were calculated using the non-overlapping trapezoid method. Spearman rank correlations were used to compare peak insulin concentration from the OGTT with values for SI and AIRg obtained from the FSIGT. Statistical significance was accepted when P < 0.05. All values are expressed as mean ± SEM unless otherwise indicated.

2.5 Results

2.5.1 Animals

All horses and ponies remained clinically healthy throughout the study and no individuals showed signs of laminitis. There were no differences detected between breed groups in age (P = 0.83) or BCS (P = 0.37). Cresty neck score was higher in Andalusians compared with Standardbreds (P = 0.039), but no differences were detected in other pair-wise comparisons (P > 0.28). Mean (± SEM) plasma cortisol concentrations measured at 0 min during the FSIGT were 90.2 ± 9.2 nmol/L for Standardbreds, 92.9 ± 8.1 for ponies and 88.3 ± 7.9 for Andalusians, with no difference detected between breed groups (P = 0.93).
2.5.2 Oral glucose tolerance test

All meals were consumed entirely. The median (range) time to consume the meal was 22 min (14 to 30) for Standardbreds, 19 min (12 to 30) for ponies, and 21 min (15 to 30) for Andalusians, with no difference detected between groups ($P = 0.54$). The glucose and insulin curves obtained from the OGTT are shown in Figure 1. Variables for glucose and insulin from the OGTT are listed in Table 1. There were no differences detected in baseline glucose, peak glucose, or glucose AUC between breed groups. There were no differences detected in baseline insulin concentrations; however, there were marked differences in peak insulin and insulin AUC between breed groups. Peak insulin was higher in ponies ($P = 0.003$) and Andalusians ($P = 0.004$) when compared with Standardbreds. Insulin AUC was also higher in ponies ($P = 0.009$) and Andalusians ($P = 0.004$) when compared with Standardbreds. There were no differences detected in peak insulin or insulin AUC between ponies and Andalusians ($P > 0.99$). Although the time of peak insulin concentrations appeared to differ between the pony (90 min) and Andalusian (150 min) groups, this difference was not statistically significant ($P = 0.90$).

2.5.3 Minimal model analysis of FSIGT

The glucose curves from the FSIGT were similar between groups, although the return to baseline appeared to be delayed in the Andalusian group (Figure 2A). The insulin responses were different between breed groups (Figure 2B), with the endogenous insulin response to glucose being greater in the pony and Andalusian groups compared with Standardbreds, and the AUC also appreciably increased. The FSIGT minimal model results are shown in Table 2. Mean SI was lower in Andalusians ($P < 0.001$) and ponies ($P = 0.003$) when compared with Standardbreds. The AIRg was lower in Standardbreds compared with ponies ($P < 0.001$) and Andalusians ($P = 0.009$), with no difference detected between ponies and Andalusians ($P = 0.65$).
Correlations between OGTT and FSIGT

Correlations showed that the animals with greater peak insulin concentrations during the OGTT had lower SI values and greater AIRg values (Figure 3). Peak insulin concentrations following the glucose meal were correlated with SI ($P < 0.001; r_s = -0.75$) and with AIRg ($P < 0.001; r_s = 0.73$). The insulin AUC was also correlated with SI ($P < 0.001; r_s = -0.77$).

It was not possible to determine a threshold peak OGTT insulin concentration that could be used as a diagnostic cut-off for predicting an SI less of than $1.0 \times 10^{-4}/(\text{mIU} \cdot \text{min})$, if used as an arbitrary value to indicate IR. The peak OGTT insulin concentrations in Andalusians and ponies with SI values less than $1.0 \times 10^{-4}/(\text{mIU} \cdot \text{min})$ ranged from 46.4 to 293.7 µIU/mL. These values overlapped considerably with the peak OGTT insulin concentrations of Andalusians and ponies with SI values greater than $1.0 \times 10^{-4}/(\text{mIU} \cdot \text{min})$, which ranged from 46.3 to 97.9 µIU/mL. All Standardbreds had SI values greater than $2.08 \times 10^{-4}/(\text{mIU} \cdot \text{min})$ and peak OGTT insulin concentrations less than 33.2 µIU/mL.

Discussion

This study demonstrated breed-related differences in the glucose and insulin dynamics of horses and ponies in moderate body condition. Minimal model analysis of FSIGT showed that ponies and Andalusian horses had lower SI values and higher AIRg values than Standardbred horses. A corresponding hyperinsulinaemic response to a glucose-containing meal was detected in ponies and Andalusians, but not in Standardbreds. These findings highlight the fact that breed-related predispositions to hyperinsulinaemia may occur without the presence of obesity or adaptation to high glycaemic diets. The finding that Andalusian horses were relatively insulin resistant was a particularly interesting finding, as this breed is thought to be predisposed to the development of obesity and laminitis [8].

The minimal model analysis of FSIGT has been previously applied to studies of horses which evaluated the effects of diet, adiposity, exercise and endotoxaemia on glucose and insulin dynamics [3,4,23-26]. The index of SI quantifies the ability of insulin to promote glucose disposal and inhibit glycolysis [22]. When compared to the reference range reported in a previous study of 46 healthy horses [27], the mean SI for Standardbreds in our study was within the highest quintile, indicating that this breed is very insulin sensitive. The mean SI
value of ponies in this study was higher than those reported in a population of ponies in the United Kingdom [23]; however, it may be inappropriate to compare studies due to differences in methodologies. We found the Andalusians to be relatively insulin resistant, with mean SI values lower than those previously reported in studies of non-obese light-breed horses of 1.94 x 10^{-4} \text{L/(mIU\cdot min)} to 2.90 x 10^{-4} \text{L/(mIU\cdot min)} [3,24,26]. This finding may not be unsurprising due to the perceived risk of obesity and laminitis in Andalusian horses. However, all animals were assessed in moderate body condition, indicating that inherently lower SI might play a role in the development of EMS [28].

Acute insulin response to glucose is the area under the curve between 0 and 10 min of the FSIGT [22], and quantifies the pancreatic response to the glucose challenge. A wide 95% reference interval for AIRg has been reported for this variable (67 [mIU\cdot min]/L to 805 [mIU\cdot min]/L) [27]. As expected from SI results, the mean AIRg for Standardbreds was within the lowest quintile reported by Treiber et al. [27]. Higher AIRg values were detected in ponies and Andalusians, reflecting an increased pancreatic response to the glucose challenge.

Disposition index is the multiplication product of SI by AIRg, and is an indication of whether the pancreatic insulin response is adequately compensating for a given degree of IR [22]. In this study, the mean DI for Andalusians was at the lower end of the reference range reported by Treiber et al. [27], whilst the mean DI for ponies was within the highest quintile. This finding demonstrates that the Andalusians were not compensating for reduced SI as well as ponies. Glucose effectiveness (Sg) was not different between groups in the present study, indicating that the ability of glucose to mediate its own disposal and suppress endogenous gluconeogenesis was not innately different between these breeds [29].

Several methodologies have been applied to OGTT in horses, including nasogastric intubation with glucose solutions [9], oral syringe dosing with corn syrup [14] and in-feed addition of glucose (as dextrose powder) [10,30,31]. This study used an in-feed dose of 1.5 g/kg BW glucose, which is higher than the previously published in-feed OGTT of between 0.5 and 1.0 g/kg BW glucose [10,30,31]. We selected this dose after pilot studies determined 1.5 g/kg BW to be the maximum amount of glucose that could be added to a fibre-based ration whilst retaining palatability. Despite a higher dose, the glucose and insulin responses observed in the present study were not as high as those reported for ponies in studies which used 0.75 and 1.0 g/kg BW [10,30]. A direct comparison between these studies may be
appropriate due to differences in body condition, management practices and the adaptation of animals to pasture.

In the present study, no difference was detected in the glucose response to the OGTT between breed groups; however, the plasma insulin response was four to five times higher in ponies and Andalusians when compared with Standardbreds. The increased insulin responses in the pony and Andalusian groups correlated with AIRg values, indicating that increased insulin secretion occurred following both oral and intravenous glucose challenges. The accompanying hyperinsulinaemia in animals with lower SI has been referred to as compensated IR [27]. Animals which fail to produce high levels of insulin due to pancreatic beta-cell exhaustion are described as having uncompensated IR [27]; these animals would demonstrate hyperglycaemia without the same degree of hyperinsulinaemia following a glucose challenge. The breed-related differences in postprandial insulin secretion emphasises that the measurement of both glucose and insulin responses is necessary to describe glucose metabolism in horses and ponies.

The assessment of postprandial glucose and insulin responses is important in equids due to the association between hyperinsulinaemia and laminitis. Laminitis has been induced in normal ponies and Standardbreds by maintaining high insulin concentrations for prolonged periods using a euglycaemic-hyperinsulinaemic clamp technique [32,33]. The role of insulin in the pathogenesis of laminitis is supported by observations of increased laminitic episodes in horses which graze pasture [28] – especially during periods when pasture contains abundant NSC [34]. The identification of individuals with exaggerated postprandial insulin responses using a standardised OGTT may enable better management of horses and ponies at risk of developing pasture-associated laminitis [11].

Although correlations were shown between the OGTT and FSIGT in this study, the presently described OGTT may not be a practical method to diagnose IR in the field. It was not possible to determine a peak insulin concentration that could be used as a diagnostic cut-off for predicting a particular SI value due to overlap between individual animals. The insulinaemic responses of horses and ponies to standardised amounts of oral glucose may provide an indication of IR in some animals, but further work remains to optimise these protocols if they are to be used as a surrogate for FSIGT measures of SI [13,14]. When individuals were ranked for both the peak OGTT insulin response and SI result, four individuals were considerably disparate (more than six places apart) between rankings. It may
have been better to perform the OGTT and FSIGT closer together; however, the three week interval between tests was required due to stipulations in the animal ethics approval for this study. All animals were managed identically throughout the study period: same paddocks, batch of hay, husbandry and personnel; and animals underwent the FSIGT in the same order as the OGTT. Therefore it is likely that the correlations reported in this study are valid despite the interval between tests.

An increased incidence of the EMS phenotype has been suggested in pony breeds and certain horse breeds such as Morgans, Paso Finos and Quarter Horses [6,7]. Studies of comparative physiology have shown that IR appears to be a biological adaptation that allows animals to store body fat in preparation for hibernation, migration or seasonal scarcity of forage [35]. Pony breeds have been shown to retain a degree of this adaptation, as demonstrated by seasonal changes in appetite and body mass [36]. A previous study found a dominant pattern of inheritance for the incidence of laminitis in an inbred herd of Dartmoor and Welsh ponies [5]. The genotype that affords increased metabolic efficiency has been described as “thrifty genes” [5,35]. It has been postulated that an interaction between modern environmental conditions (such as improved pastures) and the “thrifty” genotype has resulted in the absence of seasonal loss of body weight, progressive obesity and the exacerbation of IR in domesticated equids [28,37].

The majority of studies into the EMS phenotype have examined horses with a predisposition to IR or laminitis when they are already obese, making distinctions between the relevant risk factors difficult [5,6]. This study controlled for body condition, so that animals were assessed without obesity confounding the interpretation of results. Although BCS is a subjective appraisal of adiposity in the horse, scores less than six out of nine are thought to correlate with total body fat mass in a linear fashion [38], so it is unlikely that there were differences in adiposity between breed groups. Although overall body condition was not different between breed groups, Andalusians were observed to have a higher cresty neck score than Standardbreds. However, the mean score of 2.3 out of 5 (“noticeable appearance of crest”) for Andalusians was much lower than the reported cut-off score of ≥ 4 out of 5 (“crest grossly enlarged”) used to predict IR in another population of horses [39].

The ponies and Andalusians used in this study were not selected due to a clinical suspicion of IR. No animal had a history of laminitis, nor did they show any signs of the condition during the study – they were recruited solely on the basis of appropriate breed-type, body condition
and level of maturity. Blood tests were not used to screen animals for IR using proxy measurements of basal insulin concentrations [27,40]. It is possible that differences in the upbringing of individual animals contributed to the differences observed in this study. All horses and ponies were recruited to the study as adults, so to minimise the confounding effects of management, all animals were sourced from different properties. The majority of animals had been reared on unimproved native (Australian) pastures, with many receiving supplemental hay. Some of the horses and ponies had spent time in training, although none had been in training during the two years preceding the study. All animals were maintained on low-NSC hay for at least 4 wk prior to testing, to minimise the effect of diet on glucose and insulin dynamics [3,4].

Stress has been identified as a factor that may influence the results of FSIGT in horses [41]. Glucocorticoids reduce whole-body glucose utilisation [42] and exogenous dexamethasone administration has been shown to induce IR in horses [43]. In this study, there were no differences detected in the plasma cortisol concentrations of horses and ponies immediately prior to the FSIGT. This is a reflection that all animals had undergone extensive acclimatisation to the facilities, procedures and personnel involved in the study. Although plasma cortisol is only one indicator of stress responses, it is unlikely that stress played an important role in the interpretation of the FSIGT.

The breed-related differences in glucose and insulin dynamics observed in this study provides further evidence that breed must be accounted for when considering the nutritional management of horses and ponies. Further studies are required to determine how metabolic differences between breeds are influenced by environmental factors such as dietary carbohydrates, and how these factors might contribute to the development of obesity and heightened risk for laminitis.
2.7 Tables

Table 1: Plasma glucose and insulin responses (mean ± SEM) following a meal containing 1.5 g/kg BW added glucose in 8 Standardbred horses, 8 ponies and 7 Andalusian horses in moderate body condition.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standardbred</th>
<th>Pony</th>
<th>Andalusian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.0 ± 0.1</td>
<td>5.1 ± 0.2</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>Peak</td>
<td>7.2 ± 0.3</td>
<td>6.8 ± 0.2</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>AUC (min·mmol·L⁻¹)</td>
<td>331 ± 68</td>
<td>268 ± 33</td>
<td>262 ± 56</td>
</tr>
<tr>
<td>Plasma insulin (µIU/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.1 ± 0.5</td>
<td>10.5 ± 2.6</td>
<td>13.3 ± 4.3</td>
</tr>
<tr>
<td>Peak</td>
<td>21.2 ± 3.5</td>
<td>94.1 ± 29.1</td>
<td>85.3 ± 18.6</td>
</tr>
<tr>
<td>AUC (min·IU·L⁻¹)</td>
<td>3.1 ± 0.5</td>
<td>13.5 ± 3.6</td>
<td>15.0 ± 2.7</td>
</tr>
</tbody>
</table>

Abbreviation: AUC, area under the curve.

\(^{a,b}\) Values with different superscript letters indicate significant differences between groups, \(P < 0.01\).

Table 2: Minimal model analysis of the frequently-sampled intravenous glucose tolerance test (FSIGT) in 8 Standardbred horses, 8 ponies and 7 Andalusian horses in moderate body condition. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standardbred</th>
<th>Pony</th>
<th>Andalusian</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI (\times 10^{-4}) L/[mIU·min]</td>
<td>5.43 ± 0.94(^a)</td>
<td>2.12 ± 0.44(^b)</td>
<td>0.99 ± 0.18(^b)</td>
</tr>
<tr>
<td>AI Rg ([\text{mIU}\cdot\text{min}])</td>
<td>77.4 ± 15.0(^b)</td>
<td>442.0 ± 88.6(^b)</td>
<td>260.6 ± 41.7(^b)</td>
</tr>
<tr>
<td>DI (\times 10^{-6})</td>
<td>514 ± 134(^{c,d})</td>
<td>859 ± 215(^c)</td>
<td>230 ± 26(^d)</td>
</tr>
<tr>
<td>Sg (\times 10^{-2}/\text{min})</td>
<td>0.89 ± 0.33</td>
<td>1.21 ± 0.16</td>
<td>0.84 ± 0.13</td>
</tr>
</tbody>
</table>

Abbreviations: AI Rg, acute insulin response to glucose; DI, disposition index; Sg, glucose effectiveness; SI, insulin sensitivity.

Data are presented as mean ± SEM.

\(^{a,b}\) Values with different superscript letters indicate significant difference between groups \(P < 0.01\).

\(^{c,d}\) Values with different superscript letters indicate significant difference between groups \(P < 0.05\).
2.8 Figures

**Figure 1:** Plasma glucose (A) and insulin (B) concentrations (mean ± SEM) in Standardbred horses (n = 8), ponies (n = 8) and Andalusian horses (n = 7) following consumption of a meal containing 1.5 g/kg BW added glucose.
Figure 2: Plasma glucose (A) and insulin (B, inset) concentrations (mean ± SEM) in Standardbred horses (n = 8), ponies (n = 8) and Andalusian horses (n = 7) undergoing an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGT). Glucose (300 mg/kg BW) and insulin (20 mIU/kg BW) were administered intravenously at 0 and 20 min, respectively.
Figure 3: (A) Correlation between peak insulin concentration from an oral glucose tolerance test (OGTT) and the value for insulin sensitivity (SI) obtained from an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGT). (B) Correlation between peak insulin concentration from an OGTT and the acute insulin response to glucose (AIRg) obtained from the FSIGT.
2.9 References


Chapter 3 Effect of increased adiposity on insulin sensitivity and adipokine concentrations in horses and ponies fed a high fat diet, with or without a once daily high glycaemic meal

This chapter is presented as the author-accepted manuscript of a peer-reviewed article published in the *Equine Veterinary Journal* (2016; 48:369–373).

3.1 Overview

The central aims of this study were:

- To determine whether increased adiposity *per se* is responsible for the insulin dysregulation that is frequently observed in obese horses and ponies.
- To examine the metabolic effects of adaptation to a high fat diet, or a similar isocaloric diet containing a once daily glycaemic load.
- To evaluate adipokines and proinflammatory cytokines that have been proposed to contribute to the pathogenesis of insulin dysregulation in obese horses and ponies.

Supplementary Items 1, 2 and 3 appeared as supplementary online information.
3.2 Summary

**Reasons for study:** The relative influence of obesity and the adaptation to high-glycaemic diets on the development of insulin dysregulation in equids is unclear.

**Objectives:** To determine whether increased adiposity *per se* is responsible for the decreased insulin sensitivity (SI) often observed in obese horses, or whether a dietary glycaemic response is critically important.

**Study design:** Randomised controlled trial.

**Methods:** Eighteen horses and ponies were studied over a 20-week period. They received ad libitum hay, plus either a high-fat (low-glycaemic) diet (FAT; n=6) or a similar (isocaloric) diet containing 1.5 g/kg bwt once daily glucose (GLU; n=6) to induce obesity. A third group received a control ration (CON; n=6). Adiposity was monitored using body condition score (BCS), and total body fat mass percentage (TBFM) was determined using a deuterium oxide dilution technique. Insulin sensitivity was assessed using a frequently-sampled intravenous glucose tolerance test. Plasma concentrations of glucose, insulin, leptin, adiponectin, tumour necrosis factor-α (TNF-α) and serum amyloid A (SAA) were measured.

**Results:** The FAT and GLU groups became obese (BCS ≥ 7), whilst the CON group maintained moderate condition (BCS ≤ 6). Total body fat mass and leptin concentrations were increased in the FAT and GLU groups compared with the CON group (P<0.001 and P=0.003, respectively). Values for both insulin-dependent (SI) and insulin-independent (Sg) glucose disposal were higher in the GLU group compared with the FAT and CON groups (P=0.006 and P=0.03, respectively). There were no differences in adiponectin, TNF-α or SAA between groups (all P≥0.4).

**Conclusions:** Increased adiposity did not decrease insulin sensitivity in either the FAT or GLU diet groups, suggesting that obesity *per se* might not be responsible for the lower SI values reported in previous studies. Contrary to expectations, once daily glucose appeared to increase insulin sensitivity. Further work is required into the dietary causes of insulin resistance in equids.
3.3 Introduction

Pasture-associated laminitis is a significant cause of morbidity in domestic horse populations worldwide [1], with obesity or recent weight gain recognised to be a major risk factor [2,3]. Obese horses and ponies (exhibiting generalised or regional adiposity) often have evidence of insulin dysregulation – a recently proposed term that encompasses insulin resistance (IR), fasting hyperinsulinaemia, and exaggerated insulin responses to oral carbohydrates [4]. However, the association between obesity and insulin dysregulation in horses remains incompletely understood. It has been suggested that alterations to adipose tissue function in the obese state contributes to IR through the production of proinflammatory cytokines and biologically active hormones (adipokines) [5]. Leptin and adiponectin are two adipokines that are postulated to play a role in the pathogenesis of insulin dysregulation and warrant further investigation [6,7].

In a previous study that induced obesity in a cohort of Arabian horses, reduced insulin sensitivity (SI) and increased acute insulin response to glucose (AIRg) were reported when glucose and insulin dynamics were assessed using a frequently-sampled intravenous glucose tolerance test (FSIGT) [8]. These changes occurred when animals were provided with ‘sweet feed’ meals containing large amounts of non-structural carbohydrates (NSC). The adaptation of non-obese horses to ‘sweet feed’ meals has also been shown to decrease SI [9,10]. Diets that induce repeated episodes of hyperglycaemia and hyperinsulinaemia might contribute to IR through the down-regulation of chronically stimulated insulin receptors [11,12]. Therefore, the relative influence of increased adiposity and diet-associated hyperinsulinaemia on the development of insulin dysregulation remains unclear.

An improved understanding of the pathogenesis of equine insulin dysregulation could aid in the identification of ‘at risk’ individuals before the onset of laminitis [13]. The purpose of this study was to determine whether increased adiposity per se is responsible for the altered glucose and insulin dynamics observed in obese horses and ponies. We aimed to induce obesity by providing either a high-fat (low-glycaemic) diet, or a similar diet containing a once daily high-glycaemic meal. We hypothesised that SI (assessed using a FSIGT) would be reduced in horses and ponies after the induction of obesity, and that SI would be even lower in the animals that consumed the high-glycaemic meals.
3.4 Materials and methods

3.4.1 Animals and groups

Eighteen horses and ponies (six Standardbred horses [452 ± 17 kg], six mixed-breed ponies [290 ± 38 kg], six Andalusian-cross horses [446 ± 25 kg]) aged 5–19 years were used in this study. These breeds were chosen to provide a range of initial SI values [14]. Insulin sensitivity was not assessed prior to enrolment and no individuals had evidence of previous laminitis (including radiography). All animals were in moderate body condition at the outset of the study (median [range], 4.9 [3.8–5.8] out of 9) [15,16], and were determined to be free from pituitary pars intermedia dysfunction by clinical examination and the results of a low-dose dexamethasone suppression test [17]. The horses and ponies were kept in dirt paddocks and maintained on ad libitum pasture hay for at least 6 weeks prior to the study. All animals received routine anthelmintic, dental and farriery treatments as appropriate.

Animals were blocked by breed and randomly assigned to one of three diet groups using a random number generator, such that each diet group contained six animals total (two of each breed). Further detail of group composition is included as Supplementary Item 1. An a priori estimate of study power indicated that six animals per group were appropriate to detect a 1-unit change in SI with a power of 80%. Investigators were not blinded to diet group allocation during the study.

3.4.2 Study design and diets

The study was conducted over a 20-week period. All animals had free access to fresh water and pasture hay (sourced from a single batch; Table 1) in dirt paddocks at all times. Diet groups differed in the type and amount of supplementary feed (Table 1), with the weight of supplementary feeds adjusted to the body weight of each horse on the first day of each week. Twice daily supplementary meals were provided to each animal in individual yards (4 m x 4 m) at 0800 h and 1600 h on each day of the study, with any refusals recorded. All meals consisted of pelleted soyabean hulls (pre-soaked with water according to manufacturer’s directions) mixed with lucerne chaff. A vitamin and mineral supplement was added to the morning meal. The first diet group (CON group) received the standard meal only. The purpose of this group was to maintain a moderate BCS throughout the study to control for
seasonal and environmental influences on Week 20 results. In order to induce obesity, the second diet group (FAT group) received additional energy in the form of a mixture of liquid (canola)\textsuperscript{d} and granulated (vegetable)\textsuperscript{e} oils added to the meals. To allow for gastrointestinal adaptation, the amount of fat was gradually increased to 2 g/kg bwt by Week 20. The total ration provided approximately 200% of maintenance digestible energy (DE) requirements \cite{18}. Further detail of the provision of dietary fat is included in Supplementary Item 2. The third diet group (GLU group) received a ration isocaloric to the FAT group to also induce obesity. This group received 1.5 g/kg bwt glucose\textsuperscript{f} in the morning meal each day to produce a large glycaemic response. The dose of glucose was chosen after pilot studies determined this to be the maximum amount which was reliably consumed in a meal. A once daily glycaemic load was chosen to maximise peak postprandial glucose and insulin concentrations (subsequent glucose-containing meals might have caused a blunted insulin response due to a potential ‘second meal effect’ \cite{19}). The evening meal contained a reduced amount of the fat supplement to ensure that daily DE intake in the supplementary meals was equal between the FAT and GLU groups.

Individual hay consumption was measured at three time-points during the study: Week 0, Week 12 and Week 20. On these occasions, animals were kept in their individual yards for a 24-h period and hay intakes were accurately weighed. Group intakes of hay were estimated throughout the study.

### 3.4.3 Assessment of glucose and insulin responses

In a pilot study conducted prior to Week 0, postprandial glycaemic and insulinaemic responses to the FAT and GLU meals were evaluated in the six Standardbreds, six ponies and six Andalusians. Blood samples were collected via a jugular catheter at 30 min intervals over a 6-h period following the meal. Postprandial glycaemic and insulinaemic responses to the GLU (n=6) meals were assessed again during Week 12.

### 3.4.4 Assessment of adiposity

Body condition score (BCS) was assessed each week by a single experienced observer using a 9-point scale \cite{15,16} and body weight was measured using calibrated scales. To account for
large differences in body weight between breed groups, percentage change in body weight (ΔBW) from Week 0 values were calculated. The cresty neck score (CNS) described by Carter et al. [20] was used to determine neck crest adiposity. Total body fat mass (TBFM) was determined at Week 0 and Week 20 using a previously described deuterium oxide (D2O) dilution technique [21]. Briefly, a dose of 0.12 g/kg bwt D2Og was administered over 60 s through a temporary catheter in the left jugular vein. Heparinisedh blood samples (20 mL) were collected by venepuncture of the contralateral jugular vein immediately before and 4 h after D2O administration. Plasma samples were analysed in a commercial laboratoryi by gas isotope ratio mass spectrometry and TBFM was derived using previously described calculations [21].

3.4.5 Assessment of insulin sensitivity

Insulin sensitivity was determined using a FSIGT as previously described [9]. Briefly, horses were removed from the paddock at 0800 h and a catheter was placed in the left jugular vein. Baseline blood samples (10 mL) were collected 60, 45 and 0 min prior to a glucose bolus (300 mg/kg bwt; 40% wt/vol solution) administered through the jugular catheter. Twenty minutes after the glucose bolus, an insulin bolusj (20 mIU/kg bwt) was administered into the right jugular vein. Blood samples (10 mL) were collected at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min after the glucose bolus and immediately transferred into tubes containing lithium heparin anticoagulanth and placed on ice until centrifugation.

3.4.6 Blood sample collection

Jugular venous blood samples (20 mL) were collected from each animal immediately prior to the morning meals during Week 0 and Week 20. Samples were transferred to tubes containing lithium heparin or EDTA and placed on ice until centrifugation.
3.4.7 Plasma analysis

Blood samples were centrifuged at 1000 g for 10 min at 4°C and 1 mL aliquots of separated plasma were stored at –80°C until analysis. In all samples, plasma glucose concentrations were measured using a hexokinase colorimetric assay and insulin concentrations were measured using a radioimmunoassay previously validated for equine plasma [22]. In the Week 0 and Week 20 samples, concentrations of leptin, high-molecular weight adiponectin, tumour necrosis factor-α (TNF-α) and serum amyloid A (SAA) were determined using previously validated assays [7,23,24].

3.4.8 Data analysis

Glucose and insulin curves from the FSIGT were interpreted using MINMOD Millenium software as previously described [9]. Results included values for insulin sensitivity (SI), acute insulin response to glucose (AIRg), disposition index (DI), and glucose effectiveness (Sg). Data for outcome variables (Week 20 values) were analysed using the general linear model function in SPSS software. Diet, breed, and the interaction term were included as fixed effects, with age and sex also evaluated during the screening process. Week 0 values were included in the model as a covariate, with the exception of ΔBW. Significant main effects were compared using Fisher’s protected least significant difference test. Residual values were tested for normality using the Shapiro-Wilk test, homogeneity of variance was assessed using Levene’s test, and linearity of the covariate was assessed by the visual inspection of scatter plots. All data were reported as sample mean ± s.d., except where indicated, with P<0.05 considered significant.

3.5 Results

3.5.1 Animals and diets

All animals remained healthy throughout the study and no signs of laminitis were observed. The supplementary meals were well tolerated and meal refusals were negligible throughout the study period. Daily hay consumption (on a dry matter basis) in the FAT and GLU groups was measured to be 2.0 ± 0.2% bwt on all 3 occasions. Hay intake in the CON group was
measured to be 2.4 ± 0.2% bwt per day on all 3 occasions. Estimates of group hay intake throughout the study were consistent with these results. There was no influence of breed on individual hay consumption.

Peak postprandial glucose concentrations measured during the pilot study were 5.4 ± 0.3 and 7.4 ± 0.8 mmol/L for the FAT and GLU meals, respectively (P<0.001). Peak insulin concentrations measured during the pilot study were 11.6 ± 5.7 and 71.6 ± 30.8 mU/L for the FAT and GLU meals, respectively (P<0.001). When these responses were assessed in the GLU group during Week 12 there was no evidence of adaptation, with equivalent responses to Week 0 values (7.3 ± 0.8 mmol/L and 71.8 ± 32.6 mU/L for glucose and insulin, respectively).

For all outcome variables, the final statistical model included the fixed effects of diet, breed, and the interaction term, with Week 0 values included as a covariate (except ΔBW). Age and sex did not influence any outcome variable and were omitted from the final model. For all outcome variables, there was no effect of breed or the interaction term (all P≥0.1; Tables 2 and 3); however, it was considered (a priori) important that these effects remain in the model. Therefore, P values reported in the text represent the main effect of diet group. Further detail of the output from the statistical model is included in Supplementary Item 3.

### 3.5.2 Adiposity

Weekly measurements of BCS and ΔBW are presented in Figure 1. The FAT and GLU groups were obese (BCS ≥ 7) at Week 20, whilst the CON group remained in moderate body condition (BCS ≤ 6). Measures of adiposity at Week 0 and Week 20 are presented in Table 2. Body condition score, TBFM and CNS were significantly increased in the FAT and GLU groups compared with the CON group at Week 20 (all P<0.001). Similar increases in ΔBW were observed between diet groups (P=0.2).

### 3.5.3 Minimal model analysis

Insulin sensitivity was increased in the GLU group compared with the CON and FAT groups (P=0.006). All individuals within the GLU group (regardless of breed) exhibited higher SI
values at Week 20 than at Week 0. Glucose effectiveness was also increased in the GLU group compared with CON and FAT groups (P=0.03). There was no difference in AIRg between groups (P=0.8), whilst DI was lower in the FAT group compared with CON and GLU groups (P=0.005).

3.5.4 Plasma measurements

Leptin concentrations were significantly increased in the FAT and GLU groups compared with the CON group (Table 3; P=0.003). Resting insulin concentrations were higher (P=0.04) in the GLU group, whilst there were no differences in Week 20 glucose, adiponectin, TNF-α or SAA concentrations between diet groups (all P>0.4).

3.6 Discussion

In the present study, horses and ponies that became obese whilst consuming a high-fat (low-glycaemic) diet did not demonstrate reduced insulin sensitivity when compared with control animals. Furthermore, the SI values of horses and ponies which consumed a ration containing a once daily glycaemic load were increased despite the induction of obesity. These findings contradict our original hypothesis, which predicted that increased adiposity would be associated with a reduction in SI values. Therefore, obesity per se does not appear to be essential in the short-term induction of insulin dysregulation in horses and ponies.

Insulin sensitivity was assessed with a FSIGT, considered to be one of the most accurate quantitative methods used in the research setting [25]. Insulin sensitivity (SI), the measure of insulin-dependent glucose disposal, was increased in the GLU group, meaning that insulin was more effective at promoting glucose uptake from the bloodstream into the tissues. Insulin-independent glucose disposal (Sg) was also increased in the GLU group, meaning that glucose was more effective in promoting its own disposal. The acute insulin response to glucose (AIRg) quantifies endogenous insulin release in response to the intravenous glucose infusion, and was similar between groups. The disposition index (DI) is the multiplication product of SI x AIRg, and indicates whether AIRg is adequate relative to SI values [26]. In the present study, DI was lower in the FAT group, although the biological significance of this finding is not clear since SI values were not considered to be low.
The animals in this study were only obese for a short period of time. It therefore cannot be
discounted that long-standing obesity might play a role in the pathogenesis of insulin
dysregulation. Our timeframe was similar to a previous study that induced obesity alongside
significant decreases in SI in mature Arabian horses [8]. The Arabian horses demonstrated
slightly higher mean BCS (8.0 ± 0.7) than those reported in the FAT and GLU groups, whilst
the TBFM of the Arabian horses was also higher than those reported in the present study. A
comparison of specific TBFM values is difficult due to differences in TBFM assessment
methodology (ultrasonographic rump fat depth vs. D2O dilution). Although higher Week 20
BCS values might have been preferable, animal ethics approval prevented animals in the
present study going beyond BCS 8.

Body condition scoring was used throughout the study as a practical, non-invasive means to
assess progress. Horses and ponies in the FAT and GLU diet groups achieved a final BCS in
the ‘obese’ range (≥7 out of 9). However, this method is only semi-objective and does not
correlate with TBFM in a linear manner, especially at BCS greater than 6 out of 9 [27]. Total
body fat mass percentage was determined using a D2O dilution technique that has been
validated against post mortem dissection, and is considered to be the ‘gold standard’
measurement of TBFM in the live horse [21]. The values for TBFM reported in the present
study lie within the 95% confidence interval of the correlation with BCS reported by Dugdale
et al. [27].

Studies which have induced IR in horses (with or without concurrent obesity) have utilised
diets rich in sugar and starch, provided as multiple meals each day [8-10]. Glucose was
chosen as the NSC source in this study, as it induces higher post-prandial glucose and insulin
responses compared with fructose and inulin [28]. A study which quantified the glycaemic
and insulinaemic responses of horses and ponies to a meal containing 1.5 g/kg bwt glucose
has been previously reported by our group [14]. Glucose was provided in one meal per day to
the GLU group to maximise peak postprandial glycaemic and insulinaemic responses. We
aimed to make this peak as high as possible, and therefore chose a single meal, as a potential
‘second-meal effect’ [19] might have reduced insulin responses in the evening meal. The fat
supplement was not added to the morning glucose-containing meal to avoid modification of
the pancreatic insulin response. When the postprandial glycaemic and insulinaemic responses
to the GLU meal were assessed in Week 12, there was no evidence of adaptation (reduced
peak glucose or insulin concentrations) to the added glucose. The once daily nature of the
GLU diet might have meant that insulin concentrations were not sustained for long enough to
cause insulin receptor down-regulation. In fact, the GLU diet appeared to improve glucose and insulin dynamics, as evidenced by increased values for SI and Sg. Chronic stimulation of the pancreas by multiple ‘sweet feed’ meals per day might therefore be required to induce IR.

The FAT group consumed approximately 25% of daily DE content as fat, supporting previous findings that supplemental fat is well tolerated in horses [29]. High-fat diets are commonly used to induce IR in laboratory animal models of human type II diabetes mellitus [30]. These diets are often much higher in fat content (over 50% daily DE) than those reported in equine studies, and certainly SI was not found to be reduced in the FAT group. The similar rate of increase in BCS, and similar final TBFM in the FAT and GLU groups suggests that total ration digestibility was comparable between groups. There was no difference in daily hay intake between the FAT and GLU groups, meaning that the FAT diet did not suppress dry matter intake relative to the GLU diet.

A strength of the present study was the inclusion of a control population which maintained a moderate body condition throughout the study period. This allowed us to verify that any changes detected in the FAT and GLU groups were due to the effects of adiposity and diet, and not due to seasonal or management influences. Although measures of adiposity (TBFM, BCS, CNS) did not change in the CON group over the study period, there was an increase in body weight similar to that of the FAT and GLU groups. As total fat mass was not increased in the CON group, this interesting finding could therefore be due to an increase in gut fill (or less likely lean muscle mass). This point emphasises the need to monitor multiple morphometric measurements when assessing weight gain and loss in horses and ponies.

There are breed-related differences in the prevalence of obesity and laminitis among horses and ponies [31,32]. Standardbreds, ponies and Andalusians were enrolled to examine the effect of the study diets across a range of starting insulin sensitivities [14]. The study was underpowered to detect specific differences between breeds within each diet group, but the inclusion of these breeds was considered important in the study design to allow data to be extrapolated to the wider equine population. Although the numbers of horses in this study were small, we expected to see large decreases in SI values after the induction of obesity. It is important to note that all animals demonstrated increased SI and Sg values in the GLU group, regardless of breed.

Horses and ponies in the FAT and GLU groups demonstrated increases in plasma leptin at Week 20, at concentrations similar to those reported in previous studies of obese horses.
Leptin is secreted constitutively by adipocytes and plasma concentrations therefore reflect the adiposity of an individual. It has not been established whether a state of leptin resistance occurs in obese horses with insulin dysregulation, as has been ascribed to obese human patients with the metabolic syndrome [34,35]. Adiponectin concentrations were not different between diet groups in this study. Adiponectin has an inverse relationship with insulin concentrations in obese horses [7], and was decreased in ponies with a history of pasture-associated laminitis [36]. There were no changes in circulating TNF-α or SAA concentrations detected in this study. This finding supports recent work, in which cytokine-mediated inflammation was not associated with obesity or insulin dysregulation in horses [37]. Further work remains to fully elucidate the role of adipokines and proinflammatory cytokines in obese and IR horses.

This study demonstrates that although obesity is frequently associated with IR, increased adiposity per se may not directly impair insulin sensitivity. Increased adiposity was not associated with decreased SI when a diet containing only small amounts of NSC was used. Further studies are required to ascertain whether chronic obesity represents a different subset of risk factors for the development of insulin dysregulation. If we are to improve the management of horses and ponies at risk of laminitis, then a better understanding of the influence of diet on glucose and insulin dynamics is crucial.
### 3.7 Tables

**Table 1**: Nutrient and ingredient composition of the study diets (Week 20).

<table>
<thead>
<tr>
<th>Nutrient and Ingredient</th>
<th>Supplementary feeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hay</td>
</tr>
<tr>
<td><strong>Energy</strong></td>
<td></td>
</tr>
<tr>
<td>DE (MJ/kg feed, DM basis)</td>
<td>7.1</td>
</tr>
<tr>
<td>DE (as fed, MJ/100 kg bwt)</td>
<td>3.8</td>
</tr>
<tr>
<td><strong>Nutrient (%)</strong></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>7.7</td>
</tr>
<tr>
<td>ADF</td>
<td>46.0</td>
</tr>
<tr>
<td>NDF</td>
<td>75.8</td>
</tr>
<tr>
<td>NSC</td>
<td>9.2</td>
</tr>
<tr>
<td>WSC</td>
<td>7.3</td>
</tr>
<tr>
<td>Starch</td>
<td>1.8</td>
</tr>
<tr>
<td>Fat</td>
<td>1.8</td>
</tr>
<tr>
<td>Ash</td>
<td>5.5</td>
</tr>
<tr>
<td><strong>Ingredient (g/100 kg bwt)</strong></td>
<td></td>
</tr>
<tr>
<td>Soyabean hull pellets</td>
<td>200</td>
</tr>
<tr>
<td>Chaff</td>
<td>200</td>
</tr>
<tr>
<td>Fat supplement</td>
<td>0</td>
</tr>
<tr>
<td>Dextrose powder</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin/mineral supplement</td>
<td>6</td>
</tr>
</tbody>
</table>

All feeds were sourced from single batches and multiple samples of each ingredient combined for proximate analysis. Horses and ponies were fed either control (CON; n = 6), high fat (FAT; n = 6) or high glycaemic (GLU; n = 6) supplementary feeds divided into 2 daily meals. The GLU group received dextrose powder in the morning meal only and the fat supplement in the afternoon meal only (providing a once daily glycaemic stimulus). DE, digestible energy; DM, dry matter; CP, crude protein; ADF, acid detergent fibre; NDF, neutral detergent fibre; NSC, nonstructural carbohydrates; WSC, water-soluble carbohydrates.
Table 2: Morphometric measurements (sample mean ± s.d.) of horses and ponies fed a control (CON; n=6), high-fat (FAT; n=6) or once daily high-glycaemic (GLU; n=6) diet. The CON group maintained moderate body condition whilst FAT and GLU groups became obese over a 20-week period. P values represent the effects on Week 20 values.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week</th>
<th>Diet group</th>
<th>P value</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CON</td>
<td>FAT</td>
<td>GLU</td>
<td>Diet</td>
<td>Breed</td>
</tr>
<tr>
<td>BCS (1–9 scale)</td>
<td>0</td>
<td>5.0 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>4.9 ± 0.4</td>
<td>&lt;0.001</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5.3 ± 0.6a</td>
<td>7.3 ± 0.4b</td>
<td>7.6 ± 0.4b</td>
<td>&lt;0.001</td>
<td>0.5</td>
</tr>
<tr>
<td>TBFM (%)</td>
<td>0</td>
<td>7.6 ± 6.5</td>
<td>8.8 ± 3.3</td>
<td>8.7 ± 4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6.2 ± 3.3a</td>
<td>15.1 ± 3.3b</td>
<td>15.0 ± 4.1b</td>
<td>&lt;0.001</td>
<td>0.5</td>
</tr>
<tr>
<td>CNS (1–5 scale)</td>
<td>0</td>
<td>1.6 ± 0.6</td>
<td>1.8 ± 0.9</td>
<td>1.5 ± 0.6</td>
<td>&lt;0.001</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.7 ± 0.4a</td>
<td>3.0 ± 0.9b</td>
<td>2.5 ± 0.6b</td>
<td>&lt;0.001</td>
<td>0.5</td>
</tr>
<tr>
<td>ΔBW (%)</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>9.2 ± 3.1</td>
<td>10.8 ± 3.6</td>
<td>12.3 ± 2.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P values represent the effects on Week 20 values. BCS, body condition score [15,16]; TBFM, total body fat mass; CNS, cresty neck score [20]; ΔBW, change in bodyweight from Week 0. a,b Different superscript letters indicate significant differences between diet groups at Week 20 (P<0.05).
Table 3: Frequently-sampled intravenous glucose tolerance test (FSIGT) results and basal plasma concentrations in groups of horses and ponies fed a control (CON; n=6), high-fat (FAT; n=6) or once daily high-glycaemic (GLU; n=6) diet. The CON group maintained moderate body condition whilst FAT and GLU groups became obese over a 20-week period. Data are presented as sample mean ± s.d. P values represent the effects on Week 20 values.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week</th>
<th>CON (mg/dl)</th>
<th>FAT (mg/dl)</th>
<th>GLU (mg/dl)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI (×10⁻⁴ mIU·min⁻¹)</td>
<td>0</td>
<td>6.7 ± 2.4</td>
<td>6.9 ± 3.3</td>
<td>5.8 ± 2.2</td>
<td>0.005</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>6.7 ± 3.3</td>
<td>7.1 ± 3.4</td>
<td>6.3 ± 2.1</td>
<td>0.3</td>
</tr>
<tr>
<td>AIRg (mIU·min⁻¹/l)</td>
<td>0</td>
<td>465 ± 82</td>
<td>469 ± 92</td>
<td>462 ± 75</td>
<td>0.005</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>468 ± 82</td>
<td>472 ± 92</td>
<td>464 ± 75</td>
<td>0.3</td>
</tr>
<tr>
<td>DI (×10⁻³)</td>
<td>0</td>
<td>3.0 ± 2.0</td>
<td>6.1 ± 4.0</td>
<td>7.4 ± 5.3</td>
<td>0.005</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>3.0 ± 2.0</td>
<td>6.1 ± 4.0</td>
<td>7.4 ± 5.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Sg (×10⁻²/min)</td>
<td>0</td>
<td>1.4 ± 0.7</td>
<td>1.5 ± 0.8</td>
<td>2.6 ± 0.8</td>
<td>0.03</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>1.4 ± 0.7</td>
<td>1.5 ± 0.8</td>
<td>2.6 ± 0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>0</td>
<td>4.8 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>4.8 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Insulin (mIU/l)</td>
<td>0</td>
<td>4.0 ± 1.6</td>
<td>2.8 ± 1.3</td>
<td>4.1 ± 0.9</td>
<td>0.04</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>4.0 ± 1.6</td>
<td>2.8 ± 1.3</td>
<td>4.1 ± 0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0</td>
<td>1.5 ± 1.2</td>
<td>1.6 ± 1.3</td>
<td>1.3 ± 0.9</td>
<td>0.003</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>2.0 ± 2.1</td>
<td>2.6 ± 1.3</td>
<td>3.6 ± 3.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>0</td>
<td>4.3 ± 4.1</td>
<td>3.2 ± 2.8</td>
<td>3.6 ± 1.7</td>
<td>0.9</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>4.4 ± 3.9</td>
<td>3.1 ± 2.5</td>
<td>3.5 ± 1.6</td>
<td>0.5</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>0</td>
<td>2.1 ± 1.6</td>
<td>1.2 ± 1.6</td>
<td>0.7 ± 0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>2.0 ± 1.5</td>
<td>0.7 ± 0.5</td>
<td>0.7 ± 0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>SAA (µg/ml)</td>
<td>0</td>
<td>1.9 ± 0.9</td>
<td>0.9 ± 0.6</td>
<td>1.7 ± 1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>4.3 ± 2.1</td>
<td>5.5 ± 3.2</td>
<td>6.7 ± 2.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The CON group maintained moderate body condition whereas FAT and GLU groups became obese over a 20 week period. Data are presented as sample mean ± s.d. P values represent the effects on Week 20 values. SI, insulin sensitivity; AIRg, acute insulin response to glucose; DI, disposition index; Sg, glucose effectiveness; TNF-α, tumour necrosis factor-α; SAA, serum amyloid A. abDifferent superscript letters indicate significant difference between diet groups at Week 20 (P<0.05).
3.8 Figures

Figure 1: Body condition score (BCS; A) and percentage change in body weight (ΔBW; B) measured weekly in the control (CON; n=6), high-fat (FAT; n=6) and high-glycaemic (GLU; n=6) diet groups over a 20-week period. Points represent mean ± s.d.
3.9 Manufacturers’ addresses

aMicrosoft Excel, Microsoft Corp., Redmond, Washington, USA.
bEnergreen Nutrition, Shailer Park, Queensland, Australia.
cRanvet, East Botany, New South Wales, Australia.
dKohnke’s Own, Rouse Hill, New South Wales, Australia.
eStart to Finish, Eden Prairie, Minnesota, USA.
fValue Plus Animal Health Care, Horsley Park, New South Wales, Australia.
gCambridge Isotope Laboratories, Tewksbury, Massachusetts, USA.
hBD Vacutainer, Plymouth, UK.
iIso-Analytical Ltd., Crewe, UK.
jActrapid, Novo Nordisk A/S, Bagsvaerd, Denmark.
kCayman Chemical Co., Ann Arbor, Michigan, USA.
lCoat-A-Count, Siemens Diagnostics, Los Angeles, California, USA.
mMillipore, Billerica, Massachusetts, USA.
oThermo Fisher Scientific, Scoresby, Victoria, Australia.
opTridelta Development, Maynooth, County Kildare, Ireland.
pVersion 6.02, University of Pennsylvania, Kennett Square, Pennsylvania, USA.
qVersion 22, IBM Corp., New York, New York, USA.
3.10 References


3.11 Supplementary information

**Supplementary Item 1:** Summary of diet group composition after the randomisation of 18 horses and ponies to one of three diet groups. Animals were blocked by breed prior to randomisation to ensure an equal distribution among diet groups.

<table>
<thead>
<tr>
<th>Breed (n)</th>
<th>Sex (n)</th>
<th>Age, mean (range)</th>
<th>BCS, mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STB</td>
<td>PON</td>
<td>AND</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>FAT</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>GLU</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

CON = control ration, FAT = fat-only ration, GLU = once daily glucose ration, STB = Standardbred, PON = pony, AND = Andalusian, BCS = body condition score (Henneke et al., 1983).
Supplementary Item 2: (A) Amount of daily supplementary fat provided to the low-glycaemic (FAT; n=6) and once daily glucose (GLU; n=6) diet groups during each week of the study. (B) Estimated daily digestible energy (DE) intake for animals in the FAT, GLU and control (CON; n=6) diet groups during each week of the study*. The FAT and GLU diets were isocaloric at all times.

*DE intake was estimated by adding DE content of the supplementary feeds with that of hay (consumed at 2.0% bwt for the FAT and GLU groups; 2.4% bwt for the CON group). For the FAT group, the fat supplement was divided equally between the AM and PM meals. For the GLU group, the fat supplement was fed in the PM meal only.
Supplementary Item 3: Estimated marginal means (EMM) and estimated effects of the differences between the high-glycaemic (GLU; n=6), high-fat (FAT; n=6), and control (CON; n=6) diet groups at Week 20. For all variables, the statistical (general linear) model included the main effects of diet, breed, and the interaction term, with Week 0 values as a covariate (except ΔBW).

Morphometric measurements (reported in Table 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diet</th>
<th>EMM (± s.e.m.)</th>
<th>Comparison</th>
<th>Estimated effect (mean [95% CI])</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCS (1-9 scale)</td>
<td>GLU</td>
<td>7.6 ± 0.2</td>
<td>GLU vs. FAT</td>
<td>0.3 (-0.3, 0.9)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>FAT</td>
<td>7.3 ± 0.2</td>
<td>GLU vs. CON</td>
<td>2.4 (1.8, 3.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>5.2 ± 0.2</td>
<td>FAT vs. CON</td>
<td>2.1 (1.4, 2.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TBFM (%)</td>
<td>GLU</td>
<td>14.7 ± 0.8</td>
<td>GLU vs. FAT</td>
<td>-0.1 (-2.6, 2.4)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>FAT</td>
<td>14.8 ± 0.8</td>
<td>GLU vs. CON</td>
<td>8.0 (5.5, 10.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>6.7 ± 0.8</td>
<td>FAT vs. CON</td>
<td>8.1 (5.5, 10.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CNS (1-5 scale)</td>
<td>GLU</td>
<td>2.6 ± 0.1</td>
<td>GLU vs. FAT</td>
<td>-0.3 (-0.7, 0.1)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>FAT</td>
<td>2.9 ± 0.1</td>
<td>GLU vs. CON</td>
<td>0.8 (0.4, 1.2)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>1.7 ± 0.1</td>
<td>FAT vs. CON</td>
<td>1.1 (0.7, 1.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ΔBW (%)</td>
<td>GLU</td>
<td>12.3 ± 1.1</td>
<td>GLU vs. FAT</td>
<td>1.5 (-1.9, 5.0)</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>FAT</td>
<td>10.8 ± 1.1</td>
<td>GLU vs. CON</td>
<td>3.1 (-0.4, 6.5)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>9.2 ± 1.1</td>
<td>FAT vs. CON</td>
<td>1.5 (-1.9, 5.0)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

BCS = body condition score, TBFM = total body fat mass, CNS = cresty neck score, ΔBW = change in bodyweight from Week 0.

Minimal model variables (reported in Table 3).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diet</th>
<th>EMM (± s.e.m.)</th>
<th>Comparison</th>
<th>Estimated effect (mean [95% CI])</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI (x10⁻⁷/[mU·min])</td>
<td>GLU</td>
<td>4.7 ± 0.4</td>
<td>GLU vs. FAT</td>
<td>2.2 (1.0, 3.5)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>FAT</td>
<td>2.5 ± 0.4</td>
<td>GLU vs. CON</td>
<td>2.1 (0.8, 3.4)</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>2.6 ± 0.4</td>
<td>FAT vs. CON</td>
<td>-0.1 (-1.4, 1.2)</td>
<td>0.8</td>
</tr>
<tr>
<td>AIRg ([mU·min]/L)</td>
<td>GLU</td>
<td>183 ± 27</td>
<td>GLU vs. FAT</td>
<td>11 (-72, 95)</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>FAT</td>
<td>172 ± 27</td>
<td>GLU vs. CON</td>
<td>-19 (-112, 74)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>202 ± 27</td>
<td>FAT vs. CON</td>
<td>-30 (-120, 60)</td>
<td>0.5</td>
</tr>
<tr>
<td>DI (x10⁻²)</td>
<td>GLU</td>
<td>8.3 ± 0.9</td>
<td>GLU vs. FAT</td>
<td>5.7 (2.9, 8.8)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>FAT</td>
<td>2.4 ± 0.9</td>
<td>GLU vs. CON</td>
<td>1.4 (-1.7, 4.5)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>6.9 ± 0.9</td>
<td>FAT vs. CON</td>
<td>-4.5 (-7.6, -1.3)</td>
<td>0.01</td>
</tr>
<tr>
<td>Sg (x10⁻⁴/min)</td>
<td>GLU</td>
<td>2.6 ± 0.3</td>
<td>GLU vs. FAT</td>
<td>1.2 (0.2, 2.2)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>FAT</td>
<td>1.5 ± 0.3</td>
<td>GLU vs. CON</td>
<td>1.3 (0.3, 2.2)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>1.4 ± 0.3</td>
<td>FAT vs. CON</td>
<td>0.1 (-0.9, 1.1)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

SI = insulin sensitivity, AIRg = acute insulin response to glucose, DI = disposition index, Sg = glucose effectiveness.
### Plasma measurements (reported in Table 3)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diet</th>
<th>EMM (± s.e.m.)</th>
<th>Comparison</th>
<th>Estimated effect (mean [95% CI])</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>5.1 ± 0.2</td>
<td>GLU vs. FAT</td>
<td>0.1 (-0.5, 0.6)</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>FAT</td>
<td>5.0 ± 0.2</td>
<td>GLU vs. CON</td>
<td>-0.1 (-0.6, 0.5)</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>5.1 ± 0.2</td>
<td>FAT vs. CON</td>
<td>-0.1 (-0.7, 0.4)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td><strong>Insulin (mU/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>9.0 ± 1.3</td>
<td>GLU vs. FAT</td>
<td>5.4 (0.7, 10.2)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>FAT</td>
<td>3.6 ± 1.3</td>
<td>GLU vs. CON</td>
<td>4.6 (0.6, 8.7)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>4.5 ± 1.3</td>
<td>FAT vs. CON</td>
<td>-0.8 (-5.5, 3.8)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td><strong>Leptin (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>6.9 ± 0.8</td>
<td>GLU vs. FAT</td>
<td>0.5 (-2.0, 3.0)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>FAT</td>
<td>6.4 ± 0.8</td>
<td>GLU vs. CON</td>
<td>4.9 (2.4, 7.3)</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>2.0 ± 0.8</td>
<td>FAT vs. CON</td>
<td>4.4 (1.9, 6.8)</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td><strong>Adiponectin (µg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>3.6 ± 0.9</td>
<td>GLU vs. FAT</td>
<td>0.1 (-2.8, 3.1)</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>FAT</td>
<td>3.4 ± 0.9</td>
<td>GLU vs. CON</td>
<td>-0.4 (-3.4, 2.5)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>4.0 ± 0.9</td>
<td>FAT vs. CON</td>
<td>-0.6 (-3.6, 2.4)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td><strong>TNF-α (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>1.2 ± 0.3</td>
<td>GLU vs. FAT</td>
<td>0.4 (-0.5, 1.3)</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>FAT</td>
<td>0.7 ± 0.3</td>
<td>GLU vs. CON</td>
<td>-0.2 (-1.1, 0.8)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>1.4 ± 0.3</td>
<td>FAT vs. CON</td>
<td>-0.6 (-1.5, 0.3)</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td><strong>SAA (µg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>7.3 ± 2.7</td>
<td>GLU vs. FAT</td>
<td>3.7 (-5.5, 12.9)</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>FAT</td>
<td>3.6 ± 2.7</td>
<td>GLU vs. CON</td>
<td>1.7 (-6.6, 10.0)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>5.6 ± 2.7</td>
<td>FAT vs. CON</td>
<td>-2.0 (-11.8, 7.8)</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

TNF-α = tumour necrosis factor-α, SAA = serum amyloid A.
Supplementary Item 4: Postprandial (A) blood glucose and (B) plasma insulin concentrations for horses and ponies fed meals containing 1.5 g/kg bwt glucose (GLU; n=18 in Week 0, n=6 in Week 12) or an isocaloric amount of fat supplement (FAT; n=12 in Week 0, n=6 in Week 12). Each diet group contained equal numbers of Standardbred horses, ponies and Andalusian horses.

Comparison of area under the curve (AUC) values for blood glucose and plasma insulin concentrations for the GLU group at Week 0 and Week 12.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week 0</th>
<th>Week 12</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose AUC (min·mmol/L⁻¹)</td>
<td>412 ± 32</td>
<td>300 ± 65</td>
<td>0.13</td>
</tr>
<tr>
<td>Plasma insulin AUC (min·mU/L⁻¹)</td>
<td>11.5 ± 2.1</td>
<td>12.1 ± 3.4</td>
<td>0.59</td>
</tr>
</tbody>
</table>
Chapter 4  Effect of increased adiposity on insulin sensitivity and adipokine concentrations in different equine breeds adapted to cereal-rich or fat-rich meals

This chapter is presented as the author-accepted manuscript of a peer-reviewed article published in *The Veterinary Journal* (2016; 214:14–20).

4.1 Overview

Based on the unexpected findings reported in Chapter 3, this study modified the high-glycaemic diet to replace once daily supplemental glucose with twice daily supplemental cereal grains.

The central aims of this study were:

- To determine whether dietary glycaemic load is a more important determinant of insulin sensitivity that the development of obesity *per se*.
- To examine the metabolic effects of adaptation to fat-rich or cereal-rich diets.
- To evaluate adipokines and proinflammatory cytokines that have been proposed to contribute to the pathogenesis of insulin dysregulation in obese horses and ponies.
4.2 Abstract

The relationships between diet, obesity and insulin dysregulation in equids require further investigation due to their association with laminitis. This study examined the effect of dietary glycaemic load and increased adiposity on insulin sensitivity and adipokine concentrations in different equine breeds. Equal numbers of Standardbred horses, mixed-breed ponies and Andalusian horses were provided with ad libitum hay plus either cereal-rich (CHO; n=12), fat-rich (FAT; n=12) or control (CON; n=9) meals over 20 weeks. The isocaloric CHO and FAT diets were fed to induce obesity by gradually increasing the supplementary feeds to provide 200% of daily digestible energy requirements by Week 20. The CON group were fed a basal ration only and maintained moderate body condition.

At Week 20, the CHO and FAT groups demonstrated significantly increased body condition score, body weight, total body fat mass and plasma leptin concentrations compared with the CON group (all \( P<0.001 \)). The CHO group was found to have lower insulin sensitivity (SI; \( P<0.001 \)) and higher acute insulin response to glucose (\( P=0.002 \)) compared with the CON group. In contrast, the FAT group was no different to the controls. Ponies and Andalusians had lower SI values compared with Standardbreds, regardless of diet group (\( P=0.001 \)). Adiponectin concentrations were similar between the FAT and CON groups, but were significantly lower in the CHO group (\( P=0.010 \)). The provision of cereal-rich meals appeared to be a more important determinant of insulin sensitivity than the induction of obesity per se. Whether hypoadiponectinaemia is a cause or consequence of insulin dysregulation warrants further investigation.

4.3 Introduction

Laminitis associated with insulin dysregulation is an important cause of morbidity in domestic equine populations (Harris et al., 2006; Katz and Bailey, 2012). Insulin dysregulation is an umbrella term that includes insulin resistance, fasting hyperinsulinaemia and/or exaggerated insulin responses to oral carbohydrates (Frank and Tadros, 2014). Together with obesity (generalised or regional adiposity), insulin dysregulation has been considered to be a central component of equine metabolic syndrome (EMS) – the clinical phenotype of many equids predisposed to pasture-associated laminitis (Frank et al., 2010). Pasture-associated laminitis also occurs in non-obese horses and ponies (Bailey et al., 2007;
Geor, 2010); therefore, the link between obesity and insulin dysregulation requires further investigation. Other aspects of EMS that warrant additional study include alterations to adipokines (adipose-derived hormones such as leptin and adiponectin) and proinflammatory cytokines (Burns et al., 2010; Caltabilota et al., 2010; Wooldridge et al., 2012; Wray et al., 2013).

An apparent association between the induction of obesity and the development of hyperinsulinaemia and insulin resistance was demonstrated in a controlled study of Arabian geldings (Carter et al., 2009a). These changes occurred when horses were provided with multiple ‘sweet feed’ (cereal-rich) meals per day. The role of diet in the development of insulin dysregulation is an important consideration, because the adaptation of horses to ‘sweet feed’ meals can induce insulin resistance independent of obesity (Hoffman et al., 2003; Treiber et al., 2005). There is also evidence that weight gain can occur without reduced insulin sensitivity when horses and ponies are provided with relatively low-glycaemic rations (Quinn et al., 2008; Bamford et al., 2015a). Additionally, a once-daily oral glycaemic load appeared to improve insulin sensitivity in a group of horses and ponies (Bamford et al., 2015a). Therefore, multiple daily episodes of hyperinsulinaemia may be a necessary precedent of insulin resistance through the chronic over-stimulation of insulin receptors (Kronfeld et al., 2005; Suagee et al., 2011). The breed of animals studied also needs to be considered, as differences in the innate insulin sensitivity of different breeds will influence the insulinaemic response of an individual to oral non-structural carbohydrates (Bamford et al., 2014).

The purpose of the study reported here was to examine the relative influence of a prolonged twice-daily dietary glycaemic load, compared with an isocaloric intake of vegetable fat, on insulin sensitivity and adipokine concentrations after the induction of obesity in horses and ponies. In addition, the metabolic responses of different equine breeds were compared by enrolling three groups with previously-documented differences in innate insulin sensitivity: Standardbred horses, mixed-breed ponies and Andalusian horses (Bamford et al., 2014). We hypothesised that animals gaining weight on a cereal-rich diet would demonstrate lower insulin sensitivity than animals that gained weight on a fat-rich diet.
4.4 Materials and methods

4.4.1 Animals

Eleven Standardbred horses (9.5 ± 1.8 years, 457 ± 8 kg, BCS 5.0 ± 0.2), 11 mixed-breed ponies (9.0 ± 1.2 years, 305 ± 17 kg, BCS 5.3 ± 0.3) and 11 Andalusian-cross horses (8.3 ± 1.2 years, 475 ± 17 kg, BCS 5.5 ± 0.2) were studied. No animals demonstrated evidence of pituitary pars intermedia dysfunction when screened with a low-dose dexamethasone suppression test (McFarlane, 2011), nor did they have clinical or radiographic evidence of prior laminitis. They were kept in large dry lot paddocks with ad libitum access to fresh water and hay for at least eight weeks prior to the study. Routine hoof trimming, dental prophylaxis and anthelmintic treatments were provided as appropriate. The use of animals in this study was approved by the University of Melbourne Animal Ethics Committee (ID 1011918).

4.4.2 Study design and diets

Animals were blocked by breed and randomly assigned to one of three diet groups: a cereal-rich diet (CHO), a fat-rich diet (FAT) or a control diet (CON). The CHO and FAT groups contained 12 animals (four of each breed) and received a hypercaloric ration to induce obesity. The CON group contained nine animals (three of each breed) and received only the basal ration.

Over a 20-week study period, all animals were provided with ad libitum access to fresh water and the same hay in dry lot paddocks. Diet groups differed in the type and amount of complementary feed provided in twice-daily meals (fed at 08:00 and 16:00) on each day of the study period (Table 1). To facilitate the individual provision of meals, animals were fed in separate yards along the perimeter of the dry lot paddocks. All meals contained a base ration of soaked soyahull pellets (Maxisoy, Energreen Nutrition) and lucerne chaff, with a balanced vitamin and mineral supplement (60 mg/kg BW; Ranvet) added to the morning meals. Animals in the CHO group received additional energy in the form of micronised maize (Micrmaize, Hygain). The amount of micronised maize added to the base ration was gradually increased over the study period to allow for gastrointestinal adaptation (Figure 1). The final amount of micronised maize in the diet reached 4.55 g/kg BW (providing 3.34 g/kg BW of additional non-structural carbohydrate), with the total ration providing approximately
200% of daily digestible energy (DE) requirements (NRC, 2007). Animals in the FAT group received an isocaloric amount of supplementary vegetable fat as an equal mix (by weight) of liquid oil (Energy Gold, Kohnke’s Own) and granulated (Cool Calories, Buckeye Nutrition) fats. Mirroring the gradual increase in micronised maize for the CHO meals, supplementary vegetable fat was gradually increased in the FAT meals over the study period to allow for gastrointestinal adaptation (Figure 1). To control for seasonal and environmental influences, animals in the CON group also had *ad libitum* access to hay and received meals containing the base ration only throughout the study.

Hay consumption was accurately quantified on three separate occasions (Week 0, Week 12 and Week 20) when horses and ponies were kept in individual yards for a 24-hour period.

### 4.4.3 Assessment of adiposity

Body weight was measured weekly using calibrated scales. Percentage change from Week 0 ($\Delta$BW) was calculated to account for differences in average starting body weight between breeds. Body condition score was determined weekly by an experienced observer using a 9-point scale (Henneke et al., 1983; Kohnke, 1992). Regional adiposity along the nuchal ligament was assessed using the cresty neck score (CNS) described by Carter et al. (2009b). Total body fat mass (TBFM) was accurately determined during Week 0 and Week 20 using deuterium oxide ($D_2O$) dilution (Dugdale et al., 2011). Briefly, a dose of 0.12 g/kg BW $D_2O$ (Cambridge Isotope Laboratories) was administered through a temporary catheter in the left jugular vein. Blood samples (20 mL) were collected by venepuncture of the right jugular vein immediately before and 4 hours after $D_2O$ infusion. Syringes were weighed to determine the exact weight of $D_2O$ administered to each animal. Heparinised plasma samples were analysed using gas isotope ratio mass spectrometry (Iso-Analytical Ltd.). Total body fat mass was determined using previously described calculations (Dugdale et al., 2011).

### 4.4.4 Assessment of insulin sensitivity

Insulin sensitivity was assessed using a previously described insulin-modified frequently-sampled IV glucose tolerance test (FSIGT) during Week 0 and Week 20 (Hoffman et al., 2003). Briefly, horses and ponies were moved from the dry lot on the morning of testing and
IV catheters were placed in the left jugular vein under local anaesthesia. Blood samples were collected 60 min, 45 min and immediately before the infusion of a glucose solution (300 mg/kg BW; 40% weight/volume) through the jugular catheter. Twenty minutes later, an insulin bolus (20 mU/kg BW; Actrapid, Novo Nordisk) was delivered by venepuncture of the right jugular vein. Blood samples (10 mL) were collected 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 180, 210, 240, 270, 300, 330 and 360 minutes after the glucose infusion. Samples were transferred to tubes containing lithium heparin anticoagulant (Vacutainer, BD) and placed on ice until centrifugation.

4.4.5 Blood collection

Blood samples were collected during Week 0 and Week 20 to determine plasma concentrations of glucose, insulin, leptin, adiponectin, tumour necrosis factor-α (TNF-α) and serum amyloid A (SAA). Samples (20 mL) were collected from the left jugular vein immediately before the morning meals and transferred to tubes containing lithium heparin (for glucose, insulin, TNF-α and SAA) or EDTA (for leptin and adiponectin) anticoagulants (Vacutainer, BD). Samples were placed on ice until centrifugation.

4.4.6 Laboratory analysis

Blood samples were centrifuged (1000 g at 4°C for 10 min), with separated plasma stored at -80°C pending analysis. In all samples, glucose concentrations were measured using an enzymatic colorimetric assay (Cayman Chemical Co.) and insulin concentrations were measured using a radioimmunoassay (Coat-A-Count, Siemens Diagnostics) previously validated for equine samples (Tinworth et al., 2011). Plasma concentrations of leptin (Coat-A-Count, Siemens Diagnostics), high-molecular weight adiponectin (Millipore), TNF-α (Thermo Fisher Scientific) and SAA (Tridelta) were measured in samples from Weeks 0 and Week 20 using previously validated assays (Buff et al., 2002; Lavoie-Lamoureux et al., 2010; Wooldridge et al., 2012).
4.4.7 Data analysis

Glucose and insulin curves from the FSIGT were interpreted using MinMod Millennium software (Version 6.02; University of Pennsylvania). Values of insulin sensitivity (SI), acute insulin response to glucose (AIRg), disposition index (DI) and glucose effectiveness (Sg) were obtained (Boston et al., 2003).

Statistical analyses were performed using the general linear model function in SPSS (Version 22, IBM). Each outcome variable was evaluated using the fixed effects of diet, breed and the interaction between diet and breed. Week 0 values were included as a covariate for all variables with the exception of ΔBW. Significant main effects were compared using Fisher’s least significance difference test. Age and sex were not significant (P>0.20) for any of the variables and were therefore not included in the final model. Assumptions of the final model were checked using the Shapiro-Wilk test (normality of residual values) and Levene’s test (homogeneity of variance). Data were reported as mean ± SEM unless stated otherwise, with significance accepted when P<0.05.

4.5 Results

4.5.1 Animals and diets

All animals remained clinically healthy throughout the study period and no episodes of laminitis were observed. The study diets were well tolerated; meal refusals were negligible and there were no signs of gastrointestinal disturbance. Hay consumption (percentage of body weight on a dry matter basis) was measured to be 2.21 ± 0.06%, 2.04 ± 0.11% and 2.39 ± 0.08% for the CHO, FAT and CON groups, respectively. Hay consumption was lower for the FAT group compared with the CON group (P=0.027), but was not different between other pairwise comparisons (P=0.34). Group hay intake over the study period was consistent with the values recorded for individual consumption.
4.5.2 Adiposity

Body condition score, TBFM, CNS and ΔBW were significantly increased (all $P<0.001$) at Week 20 in the CHO and FAT groups compared with the CON group (Table 2; Figure 2). Animals in the CHO and FAT groups were considered “obese” (BCS ≥7), whereas the CON group were in “moderate” body condition (BCS ≤6). Median (range) values for CNS were 3.5 (3.0 – 4.5) for the CHO group, 3.0 (2.5 – 4.5) for the FAT group and 2.0 (1.5 – 4.0) for the CON group. No effect of breed was detected for any of the methods used to assess adiposity.

4.5.3 Insulin sensitivity

Insulin sensitivity was decreased in the CHO group relative to the FAT and CON groups (Table 3; $P<0.001$). There was no significant effect of the high-fat diet compared with the control diet. A significant effect of breed was observed, with ponies and Andalusians demonstrating lower values for SI compared with Standardbreds (Figure 3; $P=0.001$). Values for AIRg were higher in the CHO group compared with the FAT and CON groups ($P=0.002$). Glucose effectiveness was not different between diet groups, but there was a significant effect of breed, with Standardbreds demonstrating lower Sg values than ponies and Andalusians ($P=0.013$).

4.5.4 Plasma measurements

Basal glucose and insulin concentrations were not different between diet groups (Table 4). Increased adiposity resulted in higher leptin concentrations in both the CHO and FAT groups compared with the CON group ($P<0.001$). When breeds were compared, leptin concentrations in the Andalusians compared with the Standardbreds and ponies resulted in a $P$ value of 0.084. Adiponectin concentrations were found to be lower in the CHO group compared with the FAT and CON groups ($P=0.010$). Serum amyloid A concentrations were higher in the CHO group when compared with the FAT and CON groups ($P=0.009$), with no differences in TNF-α detected between groups.
4.6 Discussion

In the present study, the induction of obesity was associated with reduced insulin sensitivity in horses and ponies that consumed a cereal-rich ration. In contrast, animals that consumed an isocaloric fat-rich (low-glycaemic) ration did not exhibit a change in insulin sensitivity despite reaching levels of adiposity that did not differ significantly from the CHO group. There was a significant effect of breed across all diet groups, with ponies and Andalusians demonstrating lower insulin sensitivity compared with Standardbreds. Plasma adiponectin concentrations were reduced in the CHO group, supporting an association between hypoadiponectinaemia and insulin dysregulation in equids. These data enable a distinction to be made between the effects of dietary glycaemic load and short-term obesity on certain metabolic changes in equids. Furthermore, this study highlights the influence of breed when investigating these responses.

Insulin sensitivity was assessed using a FSIGT, which is considered to be one of the most accurate quantitative methods used by equine researchers (Firshman and Valberg, 2007). The SI parameter of the minimal model quantifies the ability of insulin to promote glucose uptake from the bloodstream. Significantly lower SI values were recorded in the CHO group compared with the FAT and CON groups. An effect of breed was also present, with ponies and Andalusians having lower SI values compared with Standardbreds. A compensatory increase in insulin secretion following the IV glucose infusion was observed as higher AIRg values in the CHO group. The disposition index (multiplication product of SI and AIRg) is used to determine the adequacy of the insulin response to a given level of insulin sensitivity, which was not detectably different between diet groups. Glucose effectiveness (Sg) quantifies the ability of glucose to promote its own removal from the bloodstream. Although not different between diet groups, Sg values were found to be lower in Standardbreds compared with ponies and Andalusians. There is some evidence that insulin-independent glucose disposal may be upregulated in animals predisposed to obesity (Hoffman et al., 2003).

The finding of reduced insulin sensitivity in the CHO group is consistent with that of Carter and colleagues (2009a), who induced obesity in a cohort of Arabian geldings using ‘sweet feed’ (cereal-rich) meals. The mean SI value reported in the present study of $1.49 \times 10^{-4}/(\text{mU} \cdot \text{min})$ is relatively modest when compared with that of the Arabians studied by Carter et al. of $0.62 \times 10^{-4}/(\text{mU} \cdot \text{min})$. This is due in part to the influence of Standardbreds within each diet group; if Standardbreds are not considered, mean SI in the present study was $0.97 \times$
10^{-4}/(mU·min). Differences in the level of adiposity may also have influenced results from the FSIGT. The Arabian horses demonstrated slightly higher mean (± SD) values for BCS (8.0 ± 0.7) than horses and ponies in the present study (7.8 ± 0.4). Total body fat mass was also higher in the Arabian horses, but a direct comparison of TBFM values is difficult due to differences in methodology between studies (ultrasonographic fat depth vs. D₂O dilution). Carter and colleagues fed approximately 200% DE requirements for 16 consecutive weeks. In contrast, we gradually increased the amount of micronized in each meal over 20 weeks, reaching 200% DE requirements for the last 2 weeks of the study. Animal ethics approval for the present study determined the cautious increase in grain over time due to the use of breeds potentially at risk of developing laminitis.

The present study was designed similarly to a previous report by our group that described the metabolic responses of horses and ponies fed either a high-fat diet or an isocaloric diet containing a once-daily glycaemic stimulus (Bamford et al., 2015a). Yielding comparable results to the present study, a decrease in SI was not detected in the high-fat group after the induction of obesity. However, there was a significant increase in SI values for the group provided with a once-daily glycaemic stimulus (as 1.5 g/kg BW dextrose) after the induction of obesity. Based on this finding, it was hypothesised that high insulin concentrations were not sustained for long enough to cause insulin receptor down-regulation, and that chronic stimulation of the pancreas by more than one cereal-rich meal per day might be required to cause a decrease in insulin sensitivity (Williams et al., 2001; Kronfeld et al., 2005; Suagee et al., 2011).

The glycaemic and insulinaemic properties of the CHO and FAT meals used in this study have been previously reported (Bamford et al., 2015a; Bamford et al., 2015b). Maize was chosen as the supplementary cereal because of its high starch content. The micronised form ensured that starch underwent as much precaecal digestion as possible, reducing the risk of hindgut disturbances that can lead to laminitis (Kronfeld and Harris, 2003; Vervuert et al., 2004). Although the CHO meals have been shown to induce robust insulinaemic responses in a previous report, an important observation was the discrepancy in responses between different breeds (Bamford et al., 2015b).

Ponies and Andalusians experience a more profound postprandial hyperinsulinaemia than Standardbreds, which is associated with differences in innate insulin sensitivity between these breeds (Bamford et al., 2014). The hyperinsulinaemia experienced by ponies and Andalusians
in the CHO group of the present study may have contributed to the lower SI values compared with the Standardbreds. However, despite relatively modest postprandial insulin responses to the CHO meal, Standardbreds exhibited lower SI values at Week 20 compared with Week 0. It is not clear whether the decrease in SI values was solely due to the effects of twice-daily postprandial hyperinsulinaemia, or whether there may be other effects of feeding cereals that are involved. Hyperinsulinaemia has been hypothesised to represent one aspect of a genetic predisposition to laminitis in horses and ponies (Harris et al., 2006; Treiber et al., 2006).

No signs of gastrointestinal upset were observed for any of the diet groups, indicating that the rate of supplementary feed increase was sufficiently cautious. Supplementary vegetable fat was well tolerated whilst providing up to 25% of daily DE in the total ration, supporting a previous finding that supplementary vegetable fat is well tolerated in the horse (Harris et al., 1999; Kronfeld et al., 2004). Hay consumption in the FAT group appeared slightly lower than the CHO group, although the difference between means was not statistically significant. The CON group was included to verify that observations in the CHO and FAT groups were due to the effects of diet and adiposity, and not related to environmental or management factors. Percentage change in body weight was increased in control animals at Week 20 (relative to Week 0) despite equivocal TBFM values. The increase in body weight without increase in adiposity in the controls may be a limitation of the study, although this finding could potentially be due to increased gut fill or more likely an increase in lean body mass in these animals. This is supported by the fact that the control diet included good quality protein from the soybean hulls and chaff; it has been previously observed that adult horses may increase rates of muscle protein synthesis in response to feeding increased protein (Urschel et al, 2011).

Leptin is an adipokine that is constitutively secreted by mature adipocytes, functioning to signal the existing state of energy balance and aid in the regulation of body weight (Jéquier, 2002). Whether a state of leptin resistance contributes to the exacerbation of obesity in horses with insulin dysregulation has not been determined. Certainly, there is a strong correlation between leptin concentrations and fat mass in horses (Buff et al., 2002; Kearns et al., 2006). In the present study, leptin mirrored adiposity; higher concentrations were present in the CHO and FAT groups compared with the CON group. Leptin was similar between the CHO and FAT groups despite differences in SI and AIRg, suggesting that leptin was reflective of fat mass and not of insulin sensitivity.
In contrast to leptin, adiponectin is often inversely proportional to adiposity (Maury and Brichard, 2010). Hypoadiponectinaemia has been postulated to play a role in the pathogenesis of several comorbidities in humans with metabolic syndrome, due to a reduction in the anti-inflammatory, anti-arthrogenic and insulin-sensitising actions of adiponectin (Fisman and Tenenbaum, 2014). Previous studies of horses have found adiponectin to be negatively correlated with basal insulin concentrations and inversely proportional to fat mass (Kearns et al., 2006; Wooldridge et al., 2012). When laminitis status was considered, previously-laminitic ponies had lower adiponectin concentrations than control ponies (Wray et al., 2013). The present study found adiponectin concentrations to be similar between the FAT and CON groups despite differences in TBFM. However, adiponectin concentrations were significantly lower in the CHO group even though leptin concentrations and TBFM were similar to the FAT group. This finding suggests that relative hypoadiponectinaemia occurred in animals with lower insulin sensitivity, without concurrent differences in leptin concentrations or TBFM. Further work is required to determine the role of adiponectin in the pathogenesis of equine insulin dysregulation.

There is conflicting information about whether obesity represents a pro-inflammatory state in the horse (Frank and Tadros, 2014). We did not detect differences in plasma TNF-α concentration between groups, supporting a previous finding that cytokine-mediated inflammation was not associated with obesity or insulin dysregulation in horses (Holbrook et al., 2012). Recent work has indicated that SAA might be a better marker of obesity-associated inflammation in horses (Suagee et al., 2013). In the present study, higher plasma concentrations of SAA were detected in the CHO group compared with the FAT and CON groups. Adiposity was similar between the CHO and FAT groups; therefore, a possible explanation for this increase could be a reduction in anti-inflammatory activity due to the relative hypoadiponectinaemia in this group. It is important to note that absolute concentrations of SAA in the CHO group were within the reference interval for horses without infectious or inflammatory conditions (Belgrave et al., 2013).

Specific recommendations for the use of high-energy providing complementary feeds in horses predisposed to laminitis cannot be made on the basis of this study. It is clear, however, that the use of this particular form of supplementary vegetable fat did not result in decreased insulin sensitivity in a population of horses and ponies that became obese. This finding may support the rationale for the use of low-glycaemic meals as an appropriate energy source in breeds predisposed to EMS that require more calories than low non-structural carbohydrate.
forage can provide. A reduction in dietary glycaemic load has also been shown to reduce basal insulin concentrations and improve insulin sensitivity in clinical cases of EMS (Morgan et al., 2015). There seems to be a threshold level of dietary non-structural carbohydrates such as starch which lead to a significant glycaemic response and sufficiently high insulin levels to lead to insulin resistance. In the present study, the feed consumed by the CON group contained relatively more starch (14.2 g per 100kg BW per meal) than the FAT group (1.6 g); however, this was still a lot less than the amount of starch consumed by the CHO group (162.3 g per 100kg BW per meal). Pilot studies showed that both the control feed and the fat-rich feed produced minimal glycaemic and insulinaemic effects, and the control feed caused no greater response than the fat-rich feed (data not shown). This probably explains why the CON group did not become more insulin resistant than the FAT group.

A limitation of the present study is that animals were only obese for a short period of time. It cannot be discounted that chronic obesity represents a different metabolic state; additional metabolic derangements may occur with long-standing obesity that predispose certain animals to endocrinopathic laminitis. Further investigation of the chronically obese equine phenotype is required.

4.7. Conclusions

The glycaemic load of the diets used in this 20-week study appeared to be a more important influence on insulin sensitivity than the induction of obesity per se. Differences in the glucose and insulin dynamics of horse and pony breeds persisted regardless of the diet consumed, with ponies and Andalusian horses less insulin sensitive than Standardbred horses. Adiponectin may play a role in equine insulin dysregulation and warrants further investigation. These data suggest that the development of obesity and insulin dysregulation may be functionally uncoupled, which is an important premise in the further study of obesity-associated disorders in equids.
4.8 Tables

**Table 1:** Proximate analysis and ingredient composition of the study diets at Week 20.

<table>
<thead>
<tr>
<th>Nutrient (%)</th>
<th>Hay</th>
<th>Supplementary feed</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHO</td>
<td>FAT</td>
<td>CON</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Energy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DE (MJ/kg feed, DM basis)</td>
<td>7.1</td>
<td>12.4</td>
<td>16.4</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>DE (as fed; MJ/100 kg BW)</td>
<td>13.1</td>
<td>13.1</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nutrient (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>7.7</td>
<td>15.6</td>
<td>14.7</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>ADF</td>
<td>46.0</td>
<td>22.1</td>
<td>27.3</td>
<td>37.9</td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>75.8</td>
<td>33.1</td>
<td>38.7</td>
<td>58.6</td>
<td></td>
</tr>
<tr>
<td>NSC</td>
<td>9.2</td>
<td>35.9</td>
<td>5.9</td>
<td>18.4</td>
<td></td>
</tr>
<tr>
<td>WSC</td>
<td>7.3</td>
<td>5.3</td>
<td>5.5</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>1.8</td>
<td>30.6</td>
<td>0.4</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>1.8</td>
<td>4.0</td>
<td>27.8</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>5.5</td>
<td>5.0</td>
<td>5.9</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td><strong>Ingredient (g/100 kg BW)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soyahull pellets</td>
<td>300</td>
<td>300</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chaff</td>
<td>300</td>
<td>300</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micronised maize</td>
<td>455</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat supplement</td>
<td>0</td>
<td>200</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin/mineral supplement</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Proximate analysis performed at Equi-Analytical Laboratories. Hay was sourced from a single batch for the duration of the study. Animals were fed either cereal-rich (CHO), fat-rich (FAT) or control (CON) supplementary feeds divided into 2 daily meals. DM, dry matter; DE, digestible energy; CP, crude protein; ADF, acid detergent fibre; NDF, neutral detergent fibre; NSC, non-structural carbohydrate; WSC, water soluble carbohydrate.
Table 2: Morphometric measurements (mean ± SEM) of horses and ponies fed a cereal-rich (CHO; n = 12), fat-rich (FAT; n = 12) or control (CON; n = 9) diet. Each diet group consisted of an equal number of Standardbred horses, mixed-breed ponies and Andalusian horses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week</th>
<th>Diet group</th>
<th></th>
<th></th>
<th>Diet</th>
<th>Breed</th>
<th>Diet x breed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CHO</td>
<td>FAT</td>
<td>CON</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCS (1–9 scale)</td>
<td>0</td>
<td>5.5 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>5.3 ± 0.3</td>
<td></td>
<td>&lt;0.001</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>7.8 ± 0.1a</td>
<td>7.4 ± 0.1a</td>
<td>5.6 ± 0.2b</td>
<td></td>
<td>&lt;0.001</td>
<td>0.83</td>
</tr>
<tr>
<td>TBFM (%)</td>
<td>0</td>
<td>8.1 ± 0.9</td>
<td>8.9 ± 0.9</td>
<td>7.7 ± 1.9</td>
<td></td>
<td>&lt;0.001</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>16.6 ± 0.7a</td>
<td>16.0 ± 0.9</td>
<td>7.8 ± 1.4b</td>
<td></td>
<td>&lt;0.001</td>
<td>0.95</td>
</tr>
<tr>
<td>CNS (1–5 scale)</td>
<td>0</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.3</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.6 ± 0.1a</td>
<td>3.2 ± 0.2a</td>
<td>2.2 ± 0.3b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔBW (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>16.0 ± 0.7a</td>
<td>14.6 ± 0.7</td>
<td>8.1 ± 1.7b</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BCS, body condition score (Henneke et al., 1983; Kohne, 1992); TBFM, total body fat mass (determined by deuterium oxide dilution); CNS, crest neck score (Carter et al., 2009b); Δ BW, percentage change in bodyweight from Week 0. P values represent the effects on Week 20 values.

a,b Significant difference between diet groups at Week 20 (P < 0.05).
Table 3: Minimal model analysis of an insulin-modified frequently-sampled IV glucose tolerance test (FSIGT; mean ± SEM) in horses and ponies fed a cereal-rich (CHO; n = 12), fat-rich (FAT; n = 12) or control (CON; n = 9) diet. Each diet group consisted of an equal number of Standardbred horses, mixed-breed ponies and Andalusian horses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week</th>
<th>Diet group</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CHO</td>
<td>FAT</td>
</tr>
<tr>
<td>SI (x10⁻⁴/mU·min)</td>
<td>0</td>
<td>3.66 ± 0.61</td>
<td>2.48 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.49 ± 0.23a</td>
<td>2.65 ± 0.46b</td>
</tr>
<tr>
<td>AIRe (mU·min/L)</td>
<td>0</td>
<td>280 ± 58</td>
<td>289 ± 61</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>502 ± 76a</td>
<td>281 ± 43b</td>
</tr>
<tr>
<td>DI (x10⁻²)</td>
<td>0</td>
<td>9.14 ± 2.02</td>
<td>5.88 ± 1.03</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6.58 ± 0.94</td>
<td>6.53 ± 1.54</td>
</tr>
<tr>
<td>Sg (x10⁻²/min)</td>
<td>0</td>
<td>1.64 ± 0.22</td>
<td>1.80 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.22 ± 0.23</td>
<td>1.92 ± 0.20</td>
</tr>
</tbody>
</table>

SI, insulin sensitivity; AIRe, acute insulin response to glucose; DI, disposition index; Sg, glucose effectiveness. P values represent the effects on Week 20 values.

a, b Significant difference between diet groups at Week 20 (P < 0.05).

* Significant effect of breed indicative of lower SI values in ponies and Andalusaions compared with Standardbreds (P < 0.05) and lower Sg values in Standardbreds compared with ponies and Andalusaions (P < 0.05).
Table 4: Plasma concentrations (mean ± SEM) in horses and ponies fed a cereal-rich (CHO; n = 12), fat-rich (FAT; n = 12) or control (CON; n = 9) diet. Each diet group consisted of an equal number of Standardbred horses, mixed-breed ponies and Andalusian horses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week</th>
<th></th>
<th>CHO</th>
<th>FAT</th>
<th>CON</th>
<th>P Diet</th>
<th>P Breed</th>
<th>P Diet x Breed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>0</td>
<td></td>
<td>5.0 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>4.9 ± 0.2</td>
<td>0.38</td>
<td>0.19</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>5.0 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>0</td>
<td></td>
<td>6.7 ± 1.3</td>
<td>4.5 ± 0.8</td>
<td>4.1 ± 0.6</td>
<td>0.088</td>
<td>0.99</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>7.1 ± 0.6</td>
<td>6.6 ± 0.8</td>
<td>4.4 ± 0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>0</td>
<td>20</td>
<td>0.80 ± 0.18</td>
<td>1.33 ± 0.27</td>
<td>1.62 ± 0.36</td>
<td>&lt;0.001</td>
<td>0.084</td>
<td>0.33</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>0</td>
<td></td>
<td>7.07 ± 0.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.29 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.97 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>4.88 ± 0.78</td>
<td>3.99 ± 0.62</td>
<td>3.15 ± 0.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α (ng/mL)</td>
<td>0</td>
<td></td>
<td>2.15 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.14 ± 0.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.89 ± 0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.010</td>
<td>0.18</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>0.58 ± 0.26</td>
<td>0.86 ± 0.56</td>
<td>1.58 ± 1.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAA (µg/mL)</td>
<td>0</td>
<td></td>
<td>1.69 ± 0.28</td>
<td>0.93 ± 0.14</td>
<td>1.76 ± 0.49</td>
<td>0.44</td>
<td>0.99</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>6.34 ± 1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.46 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.72 ± 1.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.009</td>
<td>0.58</td>
<td>0.55</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Significant difference between diet groups at Week 20 (P < 0.05).
4.9 Figures

**Figure 1**: Amount of micronized maize (left y axis) or fat supplement (right y axis) added to the base ration in the cereal-rich (CHO) and fat-rich (FAT) meals over the study period. The fat supplement consisted of an equal mixture (by weight) of liquid oil and granulated vegetable fats. The total amount of each supplement was divided into twice-daily meals.
Figure 2: Weekly measurements (mean ± SEM) of body condition score (BCS; A) and percentage change in body weight (ΔBW; B) in the cereal-rich (CHO; n = 12), fat-rich (FAT; n = 12) and control (CON; n = 9) diet groups. Each diet group consisted of an equal number of Standardbred horses, mixed-breed ponies and Andalusian horses.
Figure 3: Insulin sensitivity (SI; A), acute insulin response to glucose (AIRg; B) and glucose effectiveness (Sg; C) determined by a FSIGT. Equal numbers of Standardbred horses (white bars), mixed-breed ponies (stippled bars) and Andalusian horses (grey bars) were fed either cereal-rich (CHO; n = 12), fat-rich (FAT; n = 12) or control (CON; n = 9) meals over 20 weeks. Data are expressed as mean ± SEM. *Indicates significant difference between diet groups (P<0.05). The model indicated a significant effect of breed for SI, with lower values for ponies and Andalusians compared with Standardbreds (P=0.001). The model indicated a significant effect of breed for Sg, with lower values for Standardbreds compared with ponies and Andalusians (P=0.013).
4.10 References


Chapter 5 Postprandial glucose, insulin and glucagon-like peptide-1 responses of different equine breeds adapted to meals containing micronized maize

This chapter is presented as the author-accepted manuscript of a peer-reviewed article published in the *Journal of Animal Science* (2015; 93:3377–3383).

5.1 Overview

Data for this study were collected during Week 12 of the diet study described in Chapter 4, which presented an opportunity to examine the enteroinsular axis of different equine breeds adapted to cereal-rich meals.

The central aim of this study was:

- To characterise the relationships between postprandial glucose, insulin and GLP-1 responses in different equine breeds adapted to cereal-rich meals.
5.2 Abstract

The enteroinsular axis is a complex system that includes the release of incretin hormones from the gut to promote the absorption and utilization of glucose after a meal. The insulinogenic effect of incretin hormones such as glucagon-like peptide-1 (GLP-1) remains poorly characterized in the horse. The aim of this study was to compare postprandial glucose, insulin, and GLP-1 responses of different equine breeds adapted to twice-daily meals containing micronized maize. Four Standardbred horses, 4 mixed-breed ponies, and 4 Andalusian-cross horses in moderate BCS (5.5 ± 0.2 out of 9) were fed meals at 0800 h and 1600 h each day. The meals contained micronized maize (mixed with soaked soyabean hulls and lucerne chaff), with the amount of maize gradually increased over 12 wk to reach a final quantity of 1.7 g/kg BW (1.1 g/kg BW starch) in each meal. Animals had ad libitum access to the same hay throughout. After 12 wk of acclimation, serial blood samples were collected from all animals over a 14-h period to measure concentrations of glucose, insulin, and GLP-1, with meals fed immediately after the 0 h and 8 h samples. Glucose area under the curve (AUC) values were similar between breed groups (P = 0.41); however, ponies and Andalusian horses exhibited significantly higher insulin AUC values after both meals compared with Standardbred horses (both P < 0.005). Postprandial GLP-1 AUC values were also significantly higher in ponies and Andalusian horses compared with Standardbred horses (breed x time interaction P < 0.001). Correlation analysis demonstrated a strong positive association between concentrations of insulin and GLP-1 over time (r_s = 0.752, P < 0.001).

The increased insulin concentrations in ponies and Andalusian horses may partly reflect lower insulin sensitivity, but could also be attributed to increased GLP-1 release. Given that hyperinsulinemia is a recognized risk factor for the development of laminitis in domestic equids, this study provides evidence that the enteroinsular axis warrants further investigation.

5.3 Introduction

Hyperinsulinemia is a risk factor for the occurrence of laminitis in grazing equids (Treiber et al., 2006; Bailey et al., 2008; Carter et al., 2009). Factors that influence postprandial glucose and insulin concentrations include the type and amount of non-structural carbohydrate (NSC; starch and water-soluble carbohydrate) consumed, the prececal digestibility of starch, and the rate of meal ingestion (Harris and Geor, 2009). When high-glycemic diets are regularly
consumed, the down-regulation of insulin receptors in target tissues can lead to a state of insulin resistance (Treiber et al., 2005). In order to adapt to decreased insulin sensitivity, postprandial insulin concentrations will increase in a self-perpetuating cycle (Frank and Tadros, 2014).

A potentiation of insulin responses when glucose is administered orally (as opposed to intravenously) is due to the release of incretin hormones from specialized enteroendocrine cells, which exert insulinogenic effects on the pancreas (Hampton et al., 1986). Glucagon-like peptide-1 (GLP-1) is an incretin hormone that has been extensively investigated in human and rodent studies, but equine data is lacking (De Graaf-Roelfsema, 2014). Genetic factors influence glucose and insulin dynamics in equids (Treiber et al., 2006). Ponies and Andalusian horses produce significantly more insulin after consuming a glucose-containing meal when compared with Standardbred horses (Bamford et al., 2014). It has not yet been determined whether an incretin response is a driver for the difference in postprandial insulin responses observed between breeds.

This study assessed postprandial concentrations of glucose, insulin, and GLP-1 in horses and ponies after a 12-wk adaptation to twice-daily meals containing micronized maize. We hypothesized that ponies and Andalusian horses would demonstrate increased postprandial insulin concentrations compared with Standardbred horses, and furthermore that there would be an associated increase in GLP-1 concentrations in these breeds.

### 5.4 Materials and methods

The study protocol was approved by the University of Melbourne Animal Ethics Committee (ID 1011918).

#### 5.4.1 Animals and diets

Four Standardbred horses (STB; 5 to 14 yr, 458 ± 17 kg, BCS 5.2 ± 0.2), 4 mixed-breed ponies (PON; 5 to 10 yr, 300 ± 19 kg, BCS 5.3 ± 0.3), and 4 Andalusian-cross horses (AND; 4 to 11 yr, 509 ± 23 kg, BCS 5.7 ± 0.3) were enrolled in this 12-wk study. Animals were kept in large dirt paddocks with *ad libitum* access to hay (sourced from a single batch; Table 1)
and fresh water throughout the study period. Meals were provided at 0800 h (AM meal) and 1600 h (PM meal) each day during the study period. To enable individual feeding at meal times, animals were moved to small separate pens along the perimeter of the paddock, with any meal refusals recorded. Each meal (Table 1) consisted of a base ration containing an equal mix (1.5 g/kg BW of each ingredient by dry weight) of soaked soyahull pellets (Maxisoy, Energreen Nutrition, Shailer Park, QLD, Australia) and lucerne chaff. A balanced vitamin and mineral supplement (60 mg/kg BW; Ranvet, East Botany, NSW, Australia) was added to the AM meal. Micronized maize (Micrmaize, Hygain, Officer, VIC, Australia) was mixed with the base ration in each meal to provide a glycemic and insulinemic stimulus. Animals were fed on a “per kg BW” basis, using the BW of each animal recorded on the first day of every week during the study. The ingredients for each meal were weighed and mixed individually to ensure the accurate provision of the study diets. The amount of grain added to each meal started at 0.7 g/kg BW and was gradually increased on a weekly basis over the 12-wk period to allow for digestive adaptation. The amount of micronized maize in each meal at wk 12 was 1.7 g/kg BW, providing 1.1 g/kg BW starch.

5.4.2 Morphometric measurements

On the first day of wk 0 and wk 12, BW was measured using calibrated horse scales and BCS was assessed by a single experienced observer using a 9-point scale (Henneke et al., 1983; Kohnke, 1992). Body weight at wk 12 was reported as the percentage change from wk 0 to account for the large difference in size between ponies and horses.

5.4.3 Sample collection

After 12 wk of meal feeding, all animals underwent a serial blood sampling procedure over a 14-h period to assess the effect of the meals on glucose, insulin, and GLP-1 concentrations. Testing occurred over a 2-d period with 6 animals tested on each day in a randomized allocation. To replicate daily husbandry practices, animals remained in the dirt paddocks overnight with ad libitum access to hay and fresh water. On the day of testing, animals were moved to their individual pens where they remained for the duration of the test. Meals were provided at the regular times of 0800 h and 1600 h. Hay and fresh water were available to the
animals at all times during the sampling period. Approximately 1 h before the AM meal, an intravenous catheter was placed in the left jugular vein of each animal under local anesthesia. A baseline sample was drawn immediately before the AM meal, with serial blood samples drawn over the 14-h period. The PM meals were provided immediately after the 8 h blood sample. Samples (10 mL at each sampling time) were placed in tubes containing lithium heparin and kept on ice until centrifugation (1,000 x g for 10 min at 4°C). Plasma was harvested and 1-mL aliquots were stored at –80°C until analysis.

5.4.4 Plasma analysis

Glucose was measured using a hexokinase colorimetric assay (Cayman Chemical Co., Ann Arbor, MI) and insulin was measured using a RIA (Coat-A-Count, Siemens Diagnostics, Los Angeles, CA) previously validated for equine plasma (Tinworth et al., 2011). For samples in which insulin concentrations were above the range of the assay (389 mU/L), dilutions were performed using insulin-depleted plasma (Borer-Weir et al., 2012). Plasma concentrations of GLP-1 were determined using an ELISA (Merck Millipore, Darnstadt, Germany) previously validated for equine plasma (Chameroy et al., 2010a). Intra-assay CV were 0.8 %, 3.8 %, and 3.7 %, and inter-assay CV were 0.9 %, 5.8 %, and 10.7 % for glucose, insulin, and GLP-1, respectively.

5.4.5 Data analysis

The area under the curve (AUC) for glucose, insulin, and GLP-1 were calculated using the non-overlapping trapezoid method (GraphPad Prism, version 6.02, GraphPad Inc., La Jolla, CA). Baseline values for AUC calculations were defined as the 0 h sample for the AM meal, and the 8 h sample for the PM meal. The AUC period for glucose and insulin was defined as the first 6 h after each meal. Concentrations of GLP-1 at the 13 h and 14 h time-points were not quantified due to the number of assay plates available; therefore, the AUC period for GLP-1 was defined as the first 4 h after each meal. An outlier that demonstrated exaggerated glucose and insulin responses (greater than 5 SD above the mean for both AM and PM meals) was identified within the PON group and excluded from statistical analysis. Data were assessed for normality with the Shapiro-Wilk test, and analyzed using a mixed-model
ANOVA (SPSS, version 22, IBM Corp., New York, NY). The model included the main
effects of breed, time (AM meal vs. PM meal), and the interaction term (breed x time), with
the random effect of individual animal. Significant main effects were compared in a pairwise
manner using Tukey’s post hoc test when appropriate. Further assumptions of the model were
checked using Levene’s test (homogeneity of variance) and Box’s test (homogeneity of
covariance). Relationships between glucose, insulin, and GLP-1 were evaluated using
Spearman’s rank-order correlation analysis of 20 individual time-points from each animal
over the 14-h sampling period. Data were reported as mean ± SEM, with significance defined
as $P < 0.05$.

5.5 Results

5.5.1 Animals

All animals remained clinically healthy, and there were no meal refusals recorded on any
occasion. Body weight increased in all individuals ($P < 0.001$), with no difference detected
between STB (12.5 ± 1.1 %), PON (15.6 ± 1.4 %), and AND (14.1 ± 1.0 %) groups ($P = 0.22$). Body condition score also increased in all individuals ($P < 0.001$), with no difference
detected between wk 12 BCS in STB (6.9 ± 0.1), PON (7.1 ± 0.3), and AND (7.3 ± 0.3)
groups ($P = 0.56$). On the day of blood sampling, meals were consumed in a similar time by
STB (16 ± 1 min), PON (15 ± 2 min), and AND (16 ± 2 min) groups ($P = 0.91$). Individual
hay consumption (% BW, DM basis) over a 24-h period was not detectably different between
STB (2.1 ± 0.1 %), PON (2.3 ± 0.2 %), and AND (2.1 ± 0.1 %) groups ($P = 0.54$). Estimated
group hay intake over the study period was consistent with the measured 24-h intake.

5.5.2 Glucose and insulin responses

Plasma concentrations of glucose and insulin over the 14-h sampling period are shown in
Figure 1, with AUC values shown in Table 2. For glucose, a significant effect of time was
detected, with lower $\text{AUC}_{\text{glucose}}$ after the PM meal relative to the AM meal ($P = 0.037$). No
effect of breed on glucose responses was detected ($P = 0.41$). For insulin, there was a
significant effect of breed, with PON and AND demonstrating significantly larger $\text{AUC}_{\text{insulin}}$
compared with STB ($P = 0.002$ and $P = 0.005$, respectively). No effect of time (AM meal vs. PM meal) on insulin responses was detected ($P = 0.87$).

### 5.5.3 GLP-1 responses

Plasma concentrations of GLP-1 over the 14-h sampling period are shown in Figure 1, with AUC values shown in Table 2. For GLP-1, there was a significant breed x time interaction ($P < 0.001$). Values for AUC$_{GLP-1}$ were higher in the PON group compared with STB during the AM meal ($P = 0.016$), and higher in the PON and AND groups compared with STB during the PM meal ($P = 0.006$ and $P < 0.001$, respectively).

### 5.5.4 Correlations

Insulin concentrations were strongly correlated with GLP-1 concentrations over the sampling period ($r_s = 0.752; P < 0.001$). Weaker correlations existed between glucose and insulin ($r_s = 0.407; P < 0.001$), and between glucose and GLP-1 ($r_s = 0.271; P < 0.001$).

### 5.5.5 Outlier pony

The outlier identified within the PON group demonstrated exaggerated postprandial concentrations of glucose (AM meal: peak 8.6 mmol/L, AUC$_{glucose}$ 12.9 mmol·h/L; PM meal: peak 10.4 mmol/L, AUC$_{glucose}$ 14.9 mmol·h/L) and insulin (AM meal: peak 387.5 mU/L, AUC$_{insulin}$ 1454 mU·h/L; PM meal: peak 506.3 mU/L, AUC$_{insulin}$ 1816 mU·h/L). However, postprandial concentrations of GLP-1 were not increased relative to the other ponies (AM meal: peak 11.8 pmol/L, AUC$_{GLP-1}$ 35.7 pmol·h/L; PM meal peak 28.9 pmol/L, AUC$_{GLP-1}$ 86.73 pmol·h/L). This pony did not demonstrate any clinical signs of laminitis despite the significant hyperinsulinemia observed.
5.6 Discussion

The evidence presented in this study supports potential breed differences in the enteroinsular axis in horses and ponies adapted to eating twice-daily meals containing micronized maize. Despite similar postprandial glucose responses between breed groups, the ponies and Andalusian horses demonstrated significantly larger insulin responses compared with Standardbred horses. Postprandial GLP-1 concentrations were positively correlated with insulin concentrations, demonstrating an association between incretin hormones and hyperinsulinemia in ponies and Andalusian horses.

Prolonged hyperinsulinemia has been shown to cause acute laminitis in ponies and Standardbred horses under experimental conditions (Asplin et al., 2007; de Laat et al., 2010). Therefore, postprandial hyperinsulinemia may be an important risk factor for laminitis in predisposed individuals (Frank and Tadros, 2014). Several important observations have supported this link, including the hyperinsulinemic response of previously-laminitic ponies to various forms of NSC (glucose, fructose, and inulin) compared with non-laminitic ponies (Bailey et al., 2007; Borer et al., 2012), and the higher incidence of laminitis in grazing equids when pastures contain increased quantities of NSC (Menzies-Gow et al., 2010). Therefore, the purpose of this work was to examine a potential reason that some types of animal (grouped here by breed) exhibit a hyperinsulinemic response. A better understanding of incretin physiology may lead to new methods to control hyperinsulinemia in horses and ponies.

Micronized maize was selected as the source of supplementary NSC in the present study to ensure that starch underwent as much prececal digestion as possible (Vervuert et al., 2004). The quantity of grain in each meal was slowly increased to reduce the risk of grain overload and hindgut disturbances that could cause laminitis (Kronfeld and Harris, 2003). Although it would have been interesting, a pre-adaptation comparison experiment was not feasible. It was considered that feeding the final amount of grain without adaptation would place the ponies and Andalusians at undue risk of laminitis. There is little evidence to suggest an appropriate time frame to adapt horses and ponies to grain meals, but there is some evidence that horses may be slow to adapt (Dyer et al., 2009). Therefore, 12 wk was considered a suitably cautious time frame for the present study. The amount of grain in each meal during wk 12 provided 1.1 g/kg BW starch, a quantity previously shown to induce robust insulin responses (Vervuert et al., 2009). Although it may be common to feed larger quantities of grain to race horses...
(Crandell et al., 1999), it would be uncommon for ponies to receive more than this amount in general practice.

Differences in postprandial insulin concentrations between Standardbred, pony, and Andalusian breed groups have been reported when animals of moderate body condition consumed a single glucose-containing meal (Bamford et al., 2014). The present study found that differences of a similar magnitude were detected between the same breed groups after a period of dietary adaptation to meals containing starch. Despite adapting to the same meals, Standardbred horses secreted much less insulin in order to deal with the same glycemic load as ponies and Andalusian horses. This further emphasizes that studies of postprandial glucose and insulin responses to various types of NSC need to account for the breeds examined; and that the application of a generic glycemic index in equine dietetics remains difficult (Harris and Geor, 2009).

Previous studies of Standardbred and light-breed horses have demonstrated reduced postprandial insulin concentrations after the second of two identical meals fed 8 h apart (Gordon and McKeever, 2005; Noble and Sillence, 2013); however, the present study did not observe this decrease. It is unclear why postprandial insulin responses were equivalent between meals in the present study, but could be due to the nature of a gradual adaptation to meal feeding, or the provision of ad libitum hay throughout the study period.

The absorption and utilization of glucose from the mammalian intestinal tract is influenced by a complex enteroinsular axis, which has been scarcely studied in horses (De Graaf-Roelfsema, 2014). Two incretin hormones have been shown to exert insulinogenic effects in other species: GLP-1 and glucose-dependent insulinotropic polypeptide (GIP). A previous study of horses confirmed the presence of an enteroinsular axis through the investigation of GIP, which is released from intestinal K cells (Duhlmeier et al., 2001). Higher glucose-to-insulin ratios were detected during an oral glucose tolerance test (associated with increased GIP concentrations), when compared with an equivalent IV glucose tolerance test. Shetland ponies were found to have greater glucose and insulin concentrations than large-breed horses; however, glucose-to-insulin ratios were similar, suggesting a comparable enteroinsular axis between these animals.

Glucagon-like peptide-1 was selected as the incretin hormone of interest in the present study. This incretin is released from intestinal L cells and acts to enhance postprandial glycemic control by increasing pancreatic β-cell responsiveness to glucose-stimulated insulin secretion.
Glucagon-like peptide-1 has been extensively investigated in human and rodent studies; however, there has been little investigation of GLP-1 in horses. One report confirmed an increase in GLP-1 concentrations during an oral sugar test, but did not detect a difference between healthy and insulin resistant groups (Chameroy et al., 2010b). Furthermore, 8 wk of overfeeding with sweet feed did not change GLP-1 concentrations in a group of obese, insulin resistant horses (Chameroy et al., 2011). In the present study, GLP-1 concentrations were found to be different between breed groups, and strongly correlated with insulin concentrations. Glucose concentrations were only weakly correlated with GLP-1, indicating that breed-related differences in GLP-1 may not be directly related to glycemic responses. These associations may be consistent with a potential role for incretin hormones in equine postprandial hyperinsulinemia.

One pony demonstrated extremely high glucose and insulin responses relative to the other individuals in the pony group. This pony did not eat faster or exhibit increased BCS relative to the other ponies, nor was it found to have an exaggerated insulin response when fed a glucose-containing meal in a previously reported study (Bamford et al., 2014). Because plasma glucose concentrations were also high, this pony might have had very efficient starch digestion and glucose absorption, or decreased uptake of glucose by the liver. The GLP-1 response was similar to other ponies, so plasma glucose was presumably the main stimulus for insulin secretion. The rate of insulin clearance by the liver will also influence the degree of postprandial hyperinsulinaemia. Therefore, the measurement of C-peptide concentrations in future studies may be indicated to assess the postprandial dynamics of insulin secretion and clearance (Toth et al., 2010).

The increased insulin and GLP-1 concentrations observed in the pony and Andalusian groups has implications for the feeding of high-glycemic meals to these (and similarly predisposed) breeds. Large amounts of NSC in the diet of these breeds may easily produce the degree of hyperinsulinemia which has been implicated in causing laminitis (de Laat et al., 2012). However, not all forms of NSC may have the same effect on GLP-1 (or indeed insulin). Because most cases of endocrinopathic laminitis are associated with grazing pasture, it would be best to assess insulin and GLP-1 responses under these conditions. However, it is difficult to feed controlled amounts of NSC as pasture. Studies of glucose and insulin dynamics in horses have therefore relied on feeding measured amounts of NSC as grain, powdered, or pelleted formulations. Further studies are required to determine the effect of different types of NSC on incretin responses in horses.
An improved understanding of the factors that contribute to equine hyperinsulinemia is of critical importance in reducing the prevalence of laminitis in domestic horse populations. This study provides evidence that the enteroinsular axis warrants further investigation.
5.7 Tables

**Table 1:** Proximate analysis of ration components (DM basis)\(^1\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Hay</th>
<th>Base ration(^2)</th>
<th>Micronized maize(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE, MJ/kg</td>
<td>7.1</td>
<td>9.5</td>
<td>16.3</td>
</tr>
<tr>
<td>CP, %</td>
<td>7.7</td>
<td>11.7</td>
<td>10.3</td>
</tr>
<tr>
<td>ADF, %</td>
<td>46.0</td>
<td>37.3</td>
<td>3.3</td>
</tr>
<tr>
<td>NDF, %</td>
<td>75.8</td>
<td>57.7</td>
<td>8.6</td>
</tr>
<tr>
<td>Nonstructural carbohydrate, %(^4)</td>
<td>9.2</td>
<td>13.1</td>
<td>73.4</td>
</tr>
<tr>
<td>Water-soluble carbohydrate, %</td>
<td>7.3</td>
<td>8.7</td>
<td>3.3</td>
</tr>
<tr>
<td>Starch, %</td>
<td>1.8</td>
<td>4.4</td>
<td>70.1</td>
</tr>
<tr>
<td>Fat, %</td>
<td>1.8</td>
<td>3.7</td>
<td>4.3</td>
</tr>
<tr>
<td>Ash, %</td>
<td>5.5</td>
<td>5.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\(^1\)Analysis performed at Equi-Analytical Laboratories, Ithaca, NY.
\(^2\)Equal mixture (by dry weight) of soy hull pellets (Maxisoy, Energreen Nutrition, Shailer Park, QLD, Australia) and lucerne chaff.
\(^3\)Micronized, Hygain, Officer, VIC, Australia.
\(^4\)Nonstructural carbohydrate = starch + water-soluble carbohydrate.
Table 2: Area under the curve (AUC) values for glucose, insulin, and glucagon-like peptide-1 (GLP-1) in Standardbred horses (STB; n=4), ponies (PON; n=3) and Andalusian horses (AND; n=4) consuming grain meals at 0800 h (AM) and 1600 h (PM)\(^1\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time</th>
<th>STB</th>
<th>PON</th>
<th>AND</th>
<th>P-value</th>
<th>Breed</th>
<th>Time</th>
<th>Breed × Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol h(^{-1})-L(^{-1})</td>
<td>AM</td>
<td>2.23 ± 0.38</td>
<td>2.78 ± 0.56</td>
<td>3.50 ± 1.05</td>
<td>0.40</td>
<td>0.037</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>1.74 ± 0.34</td>
<td>2.12 ± 0.73</td>
<td>2.98 ± 0.70</td>
<td>0.001</td>
<td>0.87</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Insulin, mU h(^{-1})-L(^{-1})</td>
<td>AM</td>
<td>68.3 ± 4.1(^a)</td>
<td>190.8 ± 29.7(^b)</td>
<td>186.0 ± 38.6(^b)</td>
<td>—</td>
<td>—</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>56.0 ± 11.1(^a)</td>
<td>226.3 ± 52.0(^b)</td>
<td>183.2 ± 14.8(^b)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>GLP-1,(^2) pmol h(^{-1})-L(^{-1})</td>
<td>AM</td>
<td>9.4 ± 2.4(^a)</td>
<td>42.8 ± 11.2(^b)</td>
<td>27.2 ± 1.4(^b)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>15.0 ± 4.5(^a)</td>
<td>63.6 ± 13.4(^b)</td>
<td>84.4 ± 1.8(^b)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a,b}\)Within a row, means without a common superscript differ (P < 0.05).

\(^1\)Each meal contained a base ration (soybean hulls and lucerne chaff) mixed with 1.7 g/kg BW micronized maize (1.1 g/kg BW starch). Low nonstructural carbohydrate hay was available throughout the sampling period.

\(^2\)Significant breed × time interaction indicates greater PM AUC\(_{\text{GLP-1}}\) compared with AM AUC\(_{\text{GLP-1}}\) for the AND group (P < 0.001). No significant effect of time occurred for STB (P = 0.15) or PON (P = 0.13) groups.
5.8 Figures

Figure 1: Plasma concentrations (mean ± SEM) of (A) glucose, (B) insulin, and (C) glucagon-like peptide-1 (GLP-1) in Standardbred horses (STB; n=4), ponies (PON; n=3), and Andalusian horses (AND; n=4) over a 14-h period. Animals were adapted to twice-daily meals containing micronized maize over a 12-wk period. Each meal contained a base ration (soyabean hulls and lucerne chaff) with 1.7 g/kg BW micronized maize (1.1 g/kg BW starch). The vertical dashed lines indicate the times of meal feeding at 0800 h and 1600 h. Low-nonstructural carbohydrate hay was available throughout the sampling period.
5.9 Literature cited


5.10 Supplementary information

Supplementary figure 1: Correlations between plasma concentrations of (A) glucose and insulin, (B) glucose and GLP-1, and (C) insulin and GLP-1, in Standardbred horses (n = 4), ponies (n = 3), and Andalusian horses (n = 4) over a 14-h sampling period.
Chapter 6  General discussion

6.1 Overview

The studies described in this thesis sought to further examine the relationships between diet, obesity and insulin dysregulation in horses and ponies. The significance of these relationships is underpinned by the inclusion of obesity and insulin dysregulation as central features within the current definition of EMS to describe animals at higher risk of developing laminitis (Frank et al., 2010). The modifying effect of diet must also be considered; both the glycaemic and caloric load of a ration can influence the development of obesity and insulin dysregulation. Laminitis represents one of the most important causes of morbidity and mortality within domestic equine populations. It is imperative that countermeasures are identified to improve the welfare of affected animals (Harris et al., 2006).

The assessment of glucose and insulin dynamics described in Chapter 2 demonstrated innate metabolic differences between certain equine breeds. This finding has important implications for the management of horses and ponies that are predisposed to the EMS phenotype, in which insulin dysregulation might occur independent of obesity or modifying dietary factors. In the subsequent diet studies conducted in Chapter 3 and Chapter 4, dietary glycaemic load appeared to influence the development of insulin dysregulation more than the presence of obesity per se. Obesity may therefore be a consequence rather than a cause of insulin dysregulation in equids. Hypoadiponectinaemia was identified in animals with reduced insulin sensitivity, suggesting that adiponectin could be a useful biomarker for insulin dysregulation. Finally, the study reported in Chapter 5 showed that GLP-1 was correlated with postprandial insulin responses in horses and ponies adapted to cereal-rich meals. Incretins could therefore represent a potential therapeutic target for the control of equine hyperinsulinaemia.

6.2 Breed differences in glucose and insulin dynamics

The study presented in Chapter 2 demonstrated innate differences in glucose and insulin dynamics between different equine breeds of moderate body condition score that were maintained on a forage-only diet (Bamford et al., 2014). Ponies and Andalusian horses exhibited lower insulin sensitivity and higher postprandial insulin responses compared with
Standardbred horses that were exposed to identical husbandry conditions. This finding has important implications for the way in which certain equine breeds might be managed due to the inherent risk of laminitis in animals with insulin dysregulation. Furthermore, the recognition of metabolic differences in animals of moderate body condition score calls into question the inclusion of obesity as an essential feature of EMS.

It has long been recognised that certain equine breeds appear predisposed to the development of obesity and laminitis; they have often been referred to as ‘easy keepers’ because of their apparently high metabolic efficiency (Johnson, 2002; Geor, 2010; Frank, 2011). From an evolutionary perspective, selection pressures may have led to the development of diverse metabolic phenotypes in equine breeds that adapted to living in different environments. There is evidence that Spanish horses and pony breeds developed from different evolutionary lines to Thoroughbred and Standardbred horses (Jansen et al., 2002). Seasonal changes in insulin sensitivity make up one part of the biological adaptation that promotes the storage of body fat in various mammalian species that migrate, hibernate or spend long periods with scarce access to forage (Johnson et al., 2013). The increased metabolic efficiency afforded by certain genotypes has been described as ‘thrifty genes’ (Neel, 1998). The identification of a dominant pattern of inheritance for laminitis in an inbred herd of ponies supports a potential thrifty genotype in equids (Treiber et al., 2006). Although, it is likely that EMS is a complex genetic trait that is the result of several inherited alleles that are influenced by the environment (McCue et al., 2015). It may therefore be the epigenetic interaction between a thrifty genotype and modern management practices, such as the provision of diets with abundant calories, which causes certain individuals to develop EMS.

In order to reduce the incidence of laminitis, the aim of any countermeasure strategy must be to identify apparently healthy horses and ponies that are genetically predisposed before they become affected (Harris et al., 2006). Current studies are being undertaken to investigate the genetic alleles that are associated with certain metabolic traits in horses with EMS, such as hyperinsulinaemia (Schultz et al., 2013). A better understanding of the way in which genetic and epigenetic factors interact with the environment to alter metabolic processes might unravel the enigmatic expression of EMS in certain horses and ponies (McCue et al., 2015). However, until genetic testing is sufficiently developed to become a practical tool, the identification of animals at risk of laminitis will continue to rely on the clinical demonstration of insulin dysregulation using oral or intravenous glucose tolerance testing procedures. Special consideration should be given to the management of breeds that are theoretically
predisposed to laminitis, including dietary measures that limit hyperinsulinaemia such as the avoidance of pasture and high-glycaemic feeds.

The study presented in Chapter 2 indicated that insulin dysregulation can occur in certain equine breeds independent of increased adiposity. Previous studies of laminitis in horses and ponies with insulin dysregulation examined animals that were already obese, making a distinction between risk factors difficult (Frank et al., 2006; Treiber et al., 2006; Carter et al., 2009c). The study described herein controlled for body condition, thereby removing this potentially confounding factor during the assessment of glucose and insulin dynamics. Obesity has been proposed to contribute to the development of insulin dysregulation in other species through the production of adipokines and proinflammatory cytokines by expansile adipose tissue, resulting in the disruption of insulin-signalling pathways (Piya et al., 2013). Unfortunately, it remains extremely challenging to measure internal fat depots or hepatic lipid accumulation in the live horse, which could differ between animals without being detected. Although currently included in the consensus definition (Frank et al., 2010), it had not been determined whether obesity is essential to the EMS phenotype.

Glucose and insulin dynamics were only assessed on a single occasion. We could not determine whether evidence of insulin dysregulation in the Andalusian horses and ponies predicted future episodes of laminitis or whether these animals were predisposed to the development of obesity. However, it was clearly demonstrated that it was possible to have significant differences in glucose and insulin dynamics independent of obesity. In order to better understand the expression of EMS, it was important to establish whether obesity is a cause or simply a consequence of insulin dysregulation in horses and ponies. The following two chapters of this thesis (Chapter 3 and Chapter 4) proceeded to further examine the relationships between diet, obesity and insulin dysregulation by inducing obesity in different groups of horses and ponies using diets of varying glycaemic loads.

6.3 Influence of diet and obesity on insulin sensitivity

The studies presented in Chapter 3 and Chapter 4 examined the influence of diet and obesity on insulin sensitivity in horses and ponies (Bamford et al., 2016a; Bamford et al., 2016b). In order to fully characterise the EMS phenotype, it is important to better understand the role of obesity. The recognition of obese horses and ponies with insulin dysregulation and a
The predisposition to laminitis may only form one part of the true phenotype, because non-obese ponies are recognised to have insulin dysregulation and a predisposition to laminitis (Bailey et al., 2007; Bailey et al., 2008; Borer et al., 2012).

The landmark study of diet-induced weight gain in horses by Carter et al. (2009b) concluded that the induction of obesity resulted in hyperinsulinaemia and compensated insulin resistance. Prior to the publication of these results, the purported association between adiposity and insulin dysregulation relied on cross-sectional studies that examined animals with pre-existing obesity (Frank et al., 2006; Treiber et al., 2006). The findings of Carter et al. (2009b) have therefore been cited as evidence for a causative relationship between obesity and insulin dysregulation in equids. What these conclusions did not clearly account for was the influence of dietary glycaemic load in the development of insulin dysregulation. The Arabian geldings studied were provided with a high concentrate diet including multiple ‘sweet feed’ meals each day. Presumably, these meals would have resulted in repeated episodes of hyperinsulinaemia. This is an important consideration, as other studies of equids have demonstrated compensated insulin resistance in young and adult horses adapted to high-glycaemic diets (Hoffman et al., 2003; Treiber et al., 2005a).

The diet study reported in Chapter 3 aimed to separate dietary glycaemic load from the development of obesity by providing two different rations to induce weight gain. One group was provided with a high fat (low glycaemic) ration to induce obesity without subjecting animals to repeated episodes hyperinsulinaemia, whilst another group consumed an isocaloric ration containing a once daily high glycaemic meal. Glucose was the NSC source added to meals in this study as it has been shown to induce higher postprandial insulin responses compared with fructose and inulin (Borer et al., 2012). In order to make the postprandial peak insulin response as robust as possible, a single high glycaemic meal was provided each day. It was thought that if multiple high glycaemic meals were provided each day, a ‘second meal effect’ might have blunted postprandial insulin responses to the evening meal (Gordon et al., 2005).

Animals from both groups gained weight at similar rates over a 20 week period, with no differences between various measures of adiposity at the conclusion of the study. The group that became obese whilst consuming the high fat (low glycaemic) diet did not demonstrate a reduction in insulin sensitivity, whilst the group that became obese whilst consuming a daily high glycaemic meal actually showed improved insulin sensitivity. These findings
contradicted the original hypothesis that increased adiposity would lead to a decrease in insulin sensitivity in both diet groups, which would be more pronounced in the group consuming the high glycaemic meals. An interesting observation in this study was the slight increase in basal insulin concentrations observed in the sugar-fed group, despite increased SI values and reduced AIRg values. Although this finding was statistically significant, the increase observed (8.5 ± 3.6 mU/L vs. 4.0 ± 2.1 mU/L for control animals; $P = 0.04$) was well below the biologically significant threshold of <30 mU/L to diagnose insulin dysregulation in unfasted horses (Hart et al., 2016). This finding adds further evidence of the unreliability of basal tests to reflect dynamic protocols.

The original design of the study reported in Chapter 3 planned to repeat the protocol with a second group of horses and ponies, the intention of which was to keep the number of animals to a manageable size in each period. Due to the unexpected findings described in the first cohort, an alternative hypothesis regarding the influence of dietary glycaemic load was proposed, and the plan to repeat the same protocol with a second cohort was abandoned. The main downside of ceasing the study after one year was that it did not have the number of animals required to allow the analysis to evaluate potential differences between breed groups. However, as the aim of the study was to examine the relationships between diet, obesity and insulin dysregulation, it was sensible to revise the study protocol and to direct the resources towards a more meaningful outcome.

The study described in Chapter 4 replaced the once daily glucose-containing meal with twice daily meals containing cereal grains. The revised hypothesis was that more sustained stimulation of the pancreas by multiple ‘sweet feed’ meals might be required to induce insulin resistance. The number of animals was increased to allow the analysis to evaluate potential differences between breed groups, and apart from the new high glycaemic diet, the design remained similar to the first diet study. Micronised maize was selected as the cereal in this study due to its high starch content and the optimal pre-caecal digestibility afforded by the micronising process (Vervuert et al., 2004).

When the second diet study was performed, the induction of obesity was associated with reduced insulin sensitivity and increased acute insulin response to glucose in the horses and ponies that consumed cereal-rich meals. Consistent with the results of the previous study, animals that became obese whilst consuming the high fat (low glycaemic) diet did not demonstrate a reduction in insulin sensitivity. These findings support the supposition that
dietary glycaemic load might influence glucose and insulin dynamics more than the presence of obesity *per se*. When the analysis was extended to look at breed, Andalusian horses and ponies demonstrated lower insulin sensitivity when compared with Standardbred horses across all diet groups. This effect was much more pronounced in the high glycaemic diet group, indicating that a genetic predisposition to insulin dysregulation could be exacerbated by a high glycaemic diet.

Postprandial glucose absorption is the primary stimulus for insulin secretion, but there are a number of stimuli that promote the release of insulin, including acetylcholine (parasympathetic nervous system), gastrointestinal hormones such as secretin and cholecystokinin, incretin hormones and amino acids such as arginine (Wilcox, 2005). It remains possible that the different arrays of fatty acids and amino acids found in cereal grains could have made a further contribution to the magnitude of insulin response, either directly or acting through incretin hormones. Although unlikely, it is also conceivable that that fatty acids within the high fat diet protected against hyperinsulinaemia as short-term obesity was induced. Further investigations of the individual diet components that promote insulin secretion in equids are warranted.

The safe use or avoidance of high glycaemic diets that can induce insulin dysregulation offers management opportunities to avoid the development of EMS in susceptible animals. The studies reported in this thesis suggest that the replacement of sugar and starch-based diets with fat and fibre-based diets would be a logical dietary strategy to avoid the insulin insensitivity that develops during chronic adaptation to sweet feed. Specific recommendations for the use of high energy complementary feeds in horses and ponies predisposed to laminitis cannot be made on the basis of these studies. It was clear, however, that the fat and fibre-based diet did not induce insulin resistance in the population of animals studied. There is recent evidence that a reduction in dietary glycaemic load alleviates basal hyperinsulinaemia and improves insulin sensitivity in clinical cases of EMS being managed by veterinarians (Morgan et al., 2016). The concept of ‘pasture-associated laminitis’ implicitly acknowledges the risk that pasture can pose to certain animals through the often unquantifiable amount of NSC that is available for consumption. In addition to the avoidance of sugar or starch-rich feeds, it has been suggested that the removal of pasture access is a necessary element of any management strategy that aims to prevent laminitis in EMS-prone animals (Frank, 2011).
The studies described in Chapter 3 and Chapter 4 only evaluated short-term obesity. It cannot be discounted that chronic obesity represents a fundamentally different metabolic state that may cause further alterations to adipokine and cytokine profiles. It is also accepted that more subtle metabolic differences may have been teased out if a larger number of animals were studied. As is frequently the case with equine studies, the husbandry requirements of these large animals often preclude the enrolment of the numbers that are possible with laboratory animals. Despite these observations, it was clear that the development of obesity per se was not associated with insulin resistance in animals that were fed a fat-rich diet.

The observation that obese horses are not always insulin resistant has been made by other authors (Johnson, 2002; Geor, 2010; Frank, 2011). Whether a true ‘metabolically healthy obese’ phenotype exists in equine populations requires further clarification; horses and ponies may have evolved to store excess adipose tissue without deleterious health consequences. The chronic adaptation to high glycaemic diets may be the trigger for a self-perpetuating cycle of insulin dysregulation, with obesity a consequence of excessive caloric intake and the anabolic effects of hyperinsulinaemia.

6.4 Adipokines and insulin dysregulation

In the diet studies presented in Chapter 3 and Chapter 4, several adipose-derived plasma analytes were measured to investigate their association with insulin dysregulation in horses and ponies following diet-induced weight gain. Whether these plasma analytes are involved in the in the pathogenesis of EMS, or whether they could become useful biomarkers of insulin dysregulation in equids, requires further investigation. In both diet studies, plasma leptin concentrations were proportional to adiposity without any differences observed between diet groups. This finding supports previous reports in which leptin concentrations were reflective of fat mass in horses (Buff et al., 2002; Kearns et al., 2006). There was no evidence in these studies to support a role for hyperleptinaemia, resulting from a state of leptin resistance, in the short-term development of equine insulin dysregulation.

Hypoadiponectinaemia was detected alongside a significant reduction in insulin sensitivity in the high-grain diet group. These data add to previous reports in which decreased adiponectin concentrations were found in obese and previously-laminitic horses and ponies (Wooldridge et al., 2012; Wray et al., 2013). A relative decrease in an adipokine that is known to have
insulin-sensitising properties does make pathophysiological sense. However, the design of these studies does not permit a distinction to be made between hypoadiponectinaemia as a cause or consequence of insulin dysregulation. Recent commentary has suggested that adiponectin might become a useful biomarker for the assessment and monitoring of clinical cases of EMS (Durham, 2016). Future studies should aim to further elucidate the role of adiponectin in equine insulin dysregulation, and to determine its potential as a diagnostic or therapeutic target.

It remains unclear whether obesity represents a proinflammatory state in the horse (Frank and Tadros, 2014). In agreement with previous work by Holbrook et al. (2012), cytokine-mediated inflammation was not detected after the induction of obesity in the present studies. Serum amyloid A, which is an acute phase protein released from the liver, has been suggested as a more sensitive marker of obesity-associated inflammation in horses (Suagee et al., 2013). Rather than a marker of obesity specifically, our studies found that SAA was higher in the grain-fed group that developed insulin dysregulation, although admittedly at concentrations reflective of very mild systemic inflammation. One hypothesis considered is that hypoadiponectinaemia led to a diminution of homeostatic anti-inflammatory activity in these animals. An alternative hypothesis is that the increase in SAA was due to a direct insult on the liver rather than being associated with mild systemic inflammation. The high starch diet may have caused small changes in hindgut permeability, leading to the absorption of minute amounts of bacterial toxins that were cleared from the portal circulation by the liver without causing systemic inflammation.

6.5 Role of incretins in postprandial hyperinsulinaemia

The study presented in Chapter 5 examined the incretin response of horses and ponies adapted to high-glycaemic meals (Bamford et al., 2015). A functional enteroinsular axis has been previously confirmed in equids subjected to oral and intravenous glucose tolerance tests (Duhlmeier et al., 2001). The aim of our study was to further characterise the incretin response of different equine breeds that exhibit varying degrees of postprandial hyperinsulinaemia. Glucagon-like peptide-1 was chosen as the incretin of interest due to the detailed investigation of this incretin in human and rodent studies, and the availability of a commercial ELISA that had been validated for equine plasma (Chameroy et al., 2010).
Ponies and Andalusian horses, which are known (from previous chapters) to exhibit postprandial hyperinsulinaemia, had higher postprandial concentrations of GLP-1 when compared with Standardbred horses. A strong positive correlation between incretin and insulin responses was observed across all breeds, suggesting that the incretin response may explain why certain breeds experience postprandial hyperinsulinaemia. Although our results were significant, only one incretin was studied in a small number of animals. Therefore, further work is required to separate the insulinotropic effect of glucose from other insulin secretagogues.

Since the publication of the study presented in Chapter 5, two other reports that examined the enteroinsular axis in equids have emerged. de Laat and colleagues (2015) measured both GLP-1 and GIP in ponies. Despite similar glucose and insulin responses to an intravenous glucose tolerance test in all animals, two distinct metabolic phenotypes (normal and hyperinsulinaemic) existed when ponies were given oral glucose. Hyperinsulinaemic ponies demonstrated higher GLP-1 concentrations, but equivocal GIP concentrations, when compared with normal ponies. This led the authors to propose that hyper-responsiveness to oral carbohydrates may be a driver, rather than a consequence, of insulin resistance. In contrast, Chameroy and colleagues (2016) did not find a significant difference between active GLP-1 concentrations in normal horses and hyperinsulinaemic horses diagnosed with EMS. The disparity between studies and the relatively small number of animals indicates that further examination of the enteroinsular axis is required.

Consideration of the role of the liver in equine insulin dysregulation is also warranted. Higher glycaemic loads may increase *de novo* lipogenesis within hepatocytes; the accumulation of intracellular lipid could cause functional disturbances in the liver, leading to decreased clearance of insulin from the blood stream. Although emphasis has been placed on increased insulin secretion as the primary driver of hyperinsulinaemia, delayed insulin clearance cannot be discounted as a contributing factor, as insulin must first pass through the portal circulation before reaching the peripheral bloodstream. A recent study did not detect an effect of delayed insulin clearance when C-peptide concentrations were measured alongside insulin responses to oral and intravenous dextrose in ponies (de Laat et al., 2016); however, further work is necessary to fully elucidate the role of hepatic function in equine insulin dysregulation.

It is essential to better understand the factors that augment the insulin response of equids in order to develop countermeasures for laminitis (Harris et al., 2006). Although the provision
of a low-glycaemic ration is the most obvious countermeasure for hyperinsulinaemia, this may not be possible when pasture access cannot be limited, low-glycaemic supplementary hay cannot be sourced or if affected individual animals cannot be managed separately to other horses. Incretins may represent an attractive therapeutic target for the pharmacological modification of postprandial hyperinsulinaemia in horses and ponies with insulin dysregulation. Further studies must aim to better characterise the dietary factors that augment the insulin response of equids. Such studies could compare the influence of NSC (simple sugars, fructans and starch) and protein composition on the incretin response.

6.6 Conclusions

Dietary glycaemic load appeared to be a more influential determinant of glucose and insulin dynamics than the induction of obesity in the horses and ponies studied. Future studies should aim to further characterise the role of obesity in the pathogenesis of equine insulin dysregulation. Although frequently present in animals with insulin dysregulation, obesity may be a modifying factor rather than an essential component of the EMS phenotype. Understanding how genetic predispositions to insulin dysregulation can be aggravated by the environment is an essential first step in the development of countermeasures to reduce the incidence of laminitis in equine populations worldwide.


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Appendix 1-1: Calculations to determine total body fat mass (TBFM) from the deuterium oxide (D₂O) dilution procedure, as described by Dugdale et al. (2011c).

The dilution space available to D₂O is calculated as:

$$D₂O\ space\ (g) = \frac{[\text{Dose}\ (g) \times \text{Pb\ amu}]}{\text{Dose\ amu}} \times \frac{(\text{Dose\ ppm} - \text{Pb\ ppm})}{(\text{Pe\ ppm} - \text{Pb\ ppm})}$$

where, Pb is baseline plasma; Pe is equilibrium plasma; Dose ppm is 998,000 (99.8 atom percent excess [APE]); Dose amu (atomic mass units) is the molar mass of the stock solution of 99.8 APE deuterium oxide (20.02 g/mol); Pb amu is calculated for each animal according to its measured deuterium abundance and an assumed constant $^{18}\text{O}$ abundance of 0.2% (a value that is usually 18.0148 g/mol).

A 4% correction factor is applied to the calculated D₂O space to account for isotopic exchange of deuterium with non-water hydrogen present in proteins, carbohydrates and possibly fats, such that:

$$\text{Corrected D₂O space} = \frac{\text{TBW}_D}{1.04}$$

where, TBW₇ is the calculated total body water mass (kg) according to D₂O dilution.

The actual body mass (BM) recorded on the day of the D₂O dilution study (BM₇) is used to determine the percentage of total body tissues that was comprised of water:

$$\%\text{TBW}_D = \left(\frac{\text{TBW}_D}{\text{BM}_D}\right) \times 100$$

Finally, the percentage of total body fat (%BF) is calculated. The adjustment is made on the assumption that stored triglycerides are anhydrous and application of the hydration factor for lean tissues (0.732):

$$\%\text{BF} = 100 - \left(\frac{\%\text{TBW}_D}{0.732}\right)$$

Total body fat mass (TBFM) is determined from the recorded BM on the day of D₂O dilution:

$$\text{TBFM (kg)} = \%\text{BF} \times \text{BM}_D$$
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