# Diagnosis and epidemiology of Equine Metabolic Syndrome in native ponies and cobs in the UK

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by

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# Abstract

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Equine metabolic syndrome (EMS) is a collection of risk factors for endocrinopathic laminitis, a painful condition of the feet which has a significant impact on the welfare of horses. The central and consistent feature of EMS is insulin dysregulation (ID), leading to hyperinsulinaemia. As a result, the diagnosis of EMS focuses on detection of ID and its associated risk factors.

Laboratory testing for EMS commonly involves measurement of insulin concentration, but immediate analysis is rarely available in clinical practice. In a cross-over study the storage of whole blood at room temperature had a clinically insignificant effect on insulin concentration compared to immediate separation and freezing. This supports the practice of posting whole blood tubes to laboratories.

At most commercial laboratories a chemiluminescent assay (CLIA) is used for measurement of insulin concentration, whereas early experimental work mostly used a radioimmunoassay (RIA), potentially affecting the translation of experimental data into clinical practice. Evaluation of the CLIA using 78 equine serum samples showed it had very good precision. There was a strong positive correlation between the RIA and CLIA, however at concentrations <100µIU/mL the CLIA results were slightly lower, and results should be interpreted accordingly.

Many diagnostic tests for EMS have been described, offering various practical and/or diagnostic advantages. Their accuracy is poorly described, however, and comparison of results problematic. One novel and four established diagnostic tests were compared. There was reasonable agreement between oral dynamic tests, despite the palatability of some feedstuffs being variable. Accuracy compared to a CGIT was adequate. Fasting basal insulin lacked sensitivity.

Management of EMS commonly involves feeding preserved forages, with soaking frequently used to reduce non-structural carbohydrate content. In the same cross-over study, soaking hay significantly reduced glycaemic and insulinaemic responses, supporting this practice for management of EMS. Insulin concentration can be substantially affected by forage type, and this should be considered during management.

The contribution of the enteroinsular axis (EIA) to ID has received increased interest in recent years. Measurement of the main hormone in the EIA, glucagon-like peptide -1 (GLP-1), following forages and oral carbohydrate tests demonstrated a functioning EIA in ponies but no consistent association with EMS status, as defined by conventional tests.

The first epidemiological study examining prevalence and risk factors for EMS was performed in 354 ponies and cobs. The prevalence of EMS was 23.3% (95%CI 17.9% – 29.8%), and risk factors identified included obesity, age, breed, a more sedentary main activity and alterations in hoof rings.

The results of this thesis will help clinicians and horse owners identify horses with EMS and implement management strategies to reduce the risk of endocrinopathic laminitis.

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# List of Abbreviations

| | |
|---|---|
| ACTH | Adrenocorticotrophic hormone |
| ANOVA | Analysis of variance |
| $AUC_g$ | Area under the curves for glucose |
| $AUC_i$ | Area under the curve for insulin |
| BCS | Body Condition Score |
| BW | Body Weight |
| CGIT | Combined glucose insulin test |
| $CGITPP_g$ | Positive phase of the glucose curve |
| CLIA | Chemiluminescent assay |
| $Cmax_g$ | Maximum concentration of glucose |
| $Cmax_i$ | Maximum concentrations of insulin |
| CNS | Cresty Neck Score |
| CSS | Charcoal-stripped serum |
| CV | Coefficient of variation |
| DM | Dry Matter |
| DPP-4 | Dipeptidyl peptidase-4 |
| EIA | Enteroinsular axis |
| EMS | Equine metabolic syndrome |
| FI | Fasting insulin |
| GLP-1 | Glucagon-like peptide -1 |
| GLP-1R | GLP-1 receptor |
| GLP-2 | Glucagon-like peptide 2 |
| GLUT-4 | Glucose transporter protein-4 |
| ID | Insulin dysregulation |

| | |
|---|---|
| IL-1 | Interleukin-1 |
| $ins_{45}$ | Insulin concentration at 45 min |
| IR | Insulin resistance |
| IRS | Insulin receptor substrate |
| ITT | Intravenous Insulin tolerance test |
| LC-MS | Liquid chromatography – mass spectrometry |
| LOA | Limits of Agreement |
| MAPK | Mitogen-activation protein kinase |
| NSC | Non-structural carbohydrate |
| OGT | Oral glucose test |
| OST | Oral sugar test |
| PI3K | Phosphoinositide 3-kinase |
| PPID | Pituitary pars intermedia dysfunction |
| RIA | Radioimmunoassay |
| SIRS | Systemic inflammatory response syndrome |
| $Tmax_i$ | Time to maximum concentration of insulin |
| TNF-α | Tumour necrosis factor-α |
| WEET | Weetabix test |
| WSC | Water-soluble carbohydrate |

# Chapter 1: Introduction and Literature review

## 1.1 Equine metabolic syndrome

An association between obesity, insulin insensitivity and laminitis was first described in the 1980s (Coffman and Colles, 1983, Jeffcott et al., 1986), but it was not until 1999 that "Laminitis, Hypothyroidism and Obesity: a peripheral cushingoid syndrome in horses" was presented as a syndrome (Johnson et al., 1999). Hypothyroidism was initially considered to be the cause, but this was later disproven by the same research group (Graves et al., 2002). Although resting thyroid hormone concentrations can be below the lower limit of the reference range during EMS (Place et al., 2010), stimulated concentrations of thyroid hormone were normal (Graves et al., 2002). Further, a lack of histological evidence in affected horses and an absence of laminitis in experimentally thyroidectomised horses all failed to support this theory (Frank et al., 2002).

The term Equine Metabolic Syndrome (EMS) was proposed in 2002 (Johnson, 2002), to describe the combination of obesity, increased risk of laminitis and an 'insulin refractory state', and to recognise the similarities to metabolic syndrome in people. The analogous condition in humans has several similar features (Johnson et al., 2009), but clinical manifestations differ. Also known as pre-diabetes, metabolic syndrome in humans frequently results in a failure of pancreatic beta cells to compensate for insulin resistance (IR), leading to chronic hyperglycaemia (Esser et al., 2014). In contrast, horses maintain compensated IR and normoglycaemia (Durham et al., 2019) and inflammatory changes in the pancreas such as amyloid deposition and fibrosis have not been found (Morgan et al., 2020). The proinflammatory state and cardiovascular consequences typical of metabolic syndrome in people are less evident in horses, where laminitis is the most significant clinical feature (Morgan et al., 2015).

Equine Metabolic Syndrome was further defined in the 2010 ACVIM consensus statement (Frank et al., 2010). It was described as a phenotype of obesity (regional or generalised), IR (hyperinsulinaemia or abnormal insulin and glucose regulation) and a predisposition to laminitis that develops in the absence of recognised causes such as grain overload, colic, colitis, or retained placenta (Frank et al., 2010). This definition was useful in encapsulating what was known about EMS at the time and provided important clinical and epidemiological insights into the development of the clinical phenotype at risk for endocrinopathic laminitis.

Although EMS is still defined as a collection of risk factors for the development of endocrinopathic laminitis, research during and since the time of the first consensus has altered its definition to recognise the central feature of insulin dysregulation (ID; Frank and Tadros, 2014). From 2006, evidence showing a direct link between hyperinsulinaemia and laminitis was beginning to accumulate in experimental (Asplin et al., 2007, de Laat et al., 2010) and field studies (Treiber et al., 2006, Walsh et al., 2009b). As further studies continued to unravel the complexity of the interrelated metabolic disturbances involved, it became abundantly clear that ID played the key role in the pathogenesis of laminitis. This was emphasised by the most recent consensus statement on EMS which states unequivocally that ID is the key central and consistent feature of EMS (Durham et al., 2019), and by the proposal of the term 'hyperinsulinaemia-associated laminitis' (Frank et al., 2020).

The 2019 consensus statement also highlighted that the epidemiology of EMS and its risk factors required more research (Durham et al., 2019). An association with obesity had been shown in several studies, but epidemiological research had not occurred to further define its role or determine the influence of other contributing factors such as age, breed and exercise. The high incidence of laminitis (Pollard et al., 2019a) and obesity among UK horses (Robin et al., 2015, Stephenson et al., 2011, Wyse et al., 2008) has highlighted EMS as a major welfare risk to UK horses (Knowles and Grieve, 2020, Owers and Chubbock, 2013), yet the prevalence of EMS had not been reported, meaning this risk is unable to be quantified. As such, further research to understand the epidemiology of this syndrome is warranted to help prevent EMS and associated laminitis.

## 1.2 Physiology of insulin secretion and function

### *1.2.1 The equine insulin molecule*

Insulin is a peptide hormone produced by the beta cells of the endocrine pancreas. It consists of 2 chains (A and B) connected by disulphide bridges, and in the horse has a molecular weight of 5748 Da (Ho et al., 2008, Wilcox, 2005). The amino acid sequence is well conserved between mammalian species, with equine insulin differing from porcine insulin at 1 amino acid residue, and from human insulin at 2 amino acid residues (Ho et al., 2008). These small differences in primary structure likely result in altered folding and tertiary structures, affecting not only the efficacy of xenogenic insulin products, but also the affinity of antibodies for the molecule in immunoassays (Tinworth et al., 2011).

### 1.2.2 Synthesis and secretion of insulin

#### 1.2.2.1 Pancreatic secretion

Insulin is synthesised as the precursor molecule proinsulin, which consists of the A and B chains that make up the final molecule, and an interconnecting chain called C-peptide. Cleavage of proinsulin results in removal of the C-peptide and uncovering of the insulin receptor binding site at the carboxyl end of the B-chain (Wahren et al., 2000, Wilcox, 2005). As this takes place in the secretory granules, equimolar amounts of C peptide and insulin are secreted into the portal circulation (Wilcox, 2005, de Graaf-Roelfsema, 2014). A substantial proportion of insulin (60% in humans (Wilcox, 2005)) is removed by hepatic first-past metabolism, in contrast to C-peptide for which hepatic metabolism is negligible.

There are several stimuli for the pancreatic secretion of insulin, the principal one being increased blood glucose concentration. Insulin-independent glucose transporters facilitate diffusion of glucose into the beta cells, and the resulting increase in intracellular glucose phosphorylation leads to secretion of insulin via K-ATP and calcium channel-dependent, and other pathways. There is a well-established biphasic insulin secretory response to intravenous glucose in humans (Wilcox, 2005). In horses, a rapid first phase of pre-formed insulin in secretory vesicles starts within 1-2 minutes of glucose infusion (Hoffman et al., 2003), but the existence and timing of the second phase varies between studies. Where identified, it appears to be later in horses than in humans, occurring after 40-50 minutes (Duehlmeier et al., 2001). Other insulin secretagogues, such as amino acids (Loos et al., 2019), can have direct secretory effects or augment the response to glucose. Acute increases in non-esterified fatty acid concentration also stimulate insulin secretion, but longer-term increases are inhibitory (Grill and Qvigstad, 2000).

Basal insulin secretion is the constant, low-level release of insulin required to maintain euglycaemia in response to hepatic glucose output (Niswender, 2011) and, in herbivores, the continuous consumption of low-glycaemic forage. It is distinct from the rapid and transient increase in insulin which occurs following a glycaemic meal.

#### 1.2.2.2 Enteroinsular Axis

The enteroinsular axis augments insulin secretion in response to enteral carbohydrate (de Graaf-Roelfsema, 2014, Duehlmeier et al., 2001). In all horses, orally administered glucose results in a greater insulinaemic response compared to an isoglycaemic dose administered intravenously (de Laat et al., 2016, Duhlmeier et al., 2001). Following a meal, the insulin

response is due in part to pancreatic stimulation by absorbed nutrients, but the presence of nutrients in the intestine also stimulates the secretion of gut-derived hormones called incretins. The two principal incretins in horses are glucagon-like peptide 1 (GLP-1) (Chameroy et al., 2010) and glucose-dependent insulinotropic peptide (GIP) (Duehlmeier et al., 2001), which are secreted by the jejunal L- and K-cells, respectively (Baggio and Drucker, 2007). Incretins have been estimated to augment post-prandial insulin secretion by the pancreas by approximately 25% (de Laat et al., 2016) in horses and 50-70% in humans (Baggio and Drucker, 2007).

Numerous extrapancreatic effects of incretins have also been demonstrated in humans, including suppression of appetite and gastric motility, and inhibition of glucagon secretion (Campbell and Drucker, 2013, Holst, 2019). These additional effects have not been investigated in horses; however, it has been demonstrated that the GLP-1 receptor (GLP-1R) is distributed across a wide range of tissues (Kheder et al., 2018). Another incretin hormone, glucagon-like peptide 2 (GLP-2) has been identified in horses (de Laat et al., 2018) and is also secreted by the L cells in response to feeding. The effects of GLP-2 are restricted to the intestine and include epithelial cell proliferation, which increases its absorptive capacity (Drucker et al., 1999).

Following secretion, GLP-1 is rapidly deactivated by dipeptidyl peptidase-4 (DPP-4) and has a half-life of less than 2 minutes in the circulation. Additional DPP-4 in the vascular endothelium of the enteric circulation results in less than 50% of secreted GLP-1 reaches the peripheral circulation (Hansen et al., 1999).

### *1.2.3 Other influences on insulin secretion*

In addition to incretins, other hormones that act as insulin secretagogues include leptin, adiponectin, secretin, gastrin, vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide (Wilcox, 2005). Inhibitory endocrine influences on insulin secretion include adrenaline, prostaglandin E and the gut-derived hormone somatostatin. (Toth et al., 2010, Wilcox, 2005).

Neural stimuli also contribute to insulin secretion, with a vagally-mediated 'cephalic phase' occurring in response to the sight, smell or ingestion of food in humans (Wiedemann et al., 2020), which, if a similar reflex exists in the horse, has implications for diagnostic testing for ID. This reflex is mediated by cholinergic muscarinic receptors on the beta cells, which when

activated cause intracellular calcium release and insulin secretion, but only in the absence of hypoglycaemia (Malaisse, 1997). Conversely, catecholamine stimulation of β-adrenergic receptors results in inhibition of insulin secretion (Wilcox, 2005).

### *1.2.4 Functions of insulin and cellular responses*

Insulin mediates its actions via binding to a transmembrane receptor. Upon binding of insulin, the insulin receptor is activated resulting in autophosphorylation of specific tyrosine residues on its cytoplasmic domain. These phosphotyrosine residues then serve as docking sites to recruit and activate a range of downstream proteins including the insulin receptor substrate (IRS) family proteins. These proteins provide a molecular scaffold onto which additional intracellular signalling molecules are recruited and in turn regulate intracellular physiology. Metabolic effects are mediated via the phosphoinositide 3-kinase (PI3K) cascade, including the metabolism of glucose, lipids, and proteins. In response to hyperglycaemia, an insulin-dependent branch of the PI3K cascade results in the translocation of glucose transporter protein-4 (GLUT4) to the cell membrane and the rapid uptake of glucose to re-establish euglycaemia (Wilcox, 2005). Some signalling by the insulin receptor has mitogenic effects, mostly mediated via mitogen-activation protein kinase (MAPK) pathways (Petersen and Shulman, 2018).

The insulin receptor has a high homology and some overlap in signalling function with the insulin-like growth factor-1 (IGF-1) receptor, resulting in 'cross talk' between insulin and IGF-1, especially at higher concentrations of insulin (Petersen and Shulman, 2018). IGF-1 receptors have predominantly mitogenic effects, and activation by insulin may account for the association between hyperinsulinaemia and some cancers in humans (Petersen and Shulman, 2018). This is also the basis of one of the proposed mechanisms of hyperinsulinaemia-associated laminitis in horses (de Laat et al., 2013). More recently, however, the same group demonstrated that insulin has a very low affinity for IGF-1 receptors in lamellar tissue, putting this hypothesis in doubt (Nanayakkara et al., 2019).

There are multiple downstream consequences of the intracellular events described above, but the major direct effect of insulin secretion is a reduction in blood glucose, amino acid and fatty acid concentrations by inhibition of their endogenous production, and promotion of their storage in liver, adipose tissue and skeletal muscle.

### 1.2.5 Insulin clearance

In normal horses, >70% of insulin secreted by the pancreas is cleared from the portal blood before it enters the peripheral circulation (Toth et al., 2010). As they are secreted in equimolar quantities, calculation of C-peptide to insulin ratio gives an assessment of insulin clearance rates. The remaining insulin which enters the peripheral circulation is cleared by the liver and kidneys (Najjar and Perdomo, 2019).

## 1.3 Pathophysiology of Insulin dysregulation

Insulin dysregulation (ID) is the key and consistent feature of EMS (Durham et al., 2019), and the terms are increasingly used interchangeably. The term ID was first introduced in 2014 (Frank and Tadros, 2014) and refers to any combination of 3 abnormalities: 1. Tissue IR (the inability of tissues to respond appropriately to insulin); 2. Basal hyperinsulinaemia; and 3. Postprandial (post carbohydrate) hyperinsulinaemia. The cause-and-effect relationship between the three components of ID, the associated clinical features of EMS (e.g., obesity and laminitis) and the abnormal cellular and metabolic processes involved is complex and requires further research in equids and humans (Frank and Tadros, 2014, Najjar and Perdomo, 2019, Shanik et al., 2008).

### 1.3.1 Insulin resistance

Insulin resistance (IR) is defined as attenuation of the biological response to normal or increased levels of insulin (Cefalu, 2001). Most commonly, this refers to impaired insulin-mediated glucose disposal, but the effects are more widespread and include increased gluconeogenesis in the liver and increased lipolysis as well as vasoactive and mitogenic effects. Insulin resistance is not a failure of insulin to interact with its target cells, but instead is mediated via altered post-receptor intracellular signalling following the binding of insulin (Petersen and Shulman, 2018). For example, in relation to glucose disposal, in a state of IR the expression of GLUT-4 within muscle cells is unchanged, but there is disruption of its translocation to the cell membrane (Waller et al., 2011). Intracellular regulation of glucose phosphorylation and oxidation and glycogen synthesis in skeletal muscle are also impaired (Abdul-Ghani and DeFronzo, 2010).

Alterations in intracellular signalling, in particular a dominance of the more mitogenic MAPK over PI3K pathway, have been shown to be associated not only with metabolic disturbances such as reduced glucose disposal, but also vascular and coagulation dysfunction in murine models and in humans (Kim et al., 2006). Research in horses has shown similar alterations, with a switch to MAPK pathway dominance following hyperinsulinaemia (Venugopal et al., 2011, Wooldridge et al., 2014) and in naturally occurring ID in horses (Morgan et al., 2016b).

Sensitivity of tissues to insulin is influenced under physiological conditions by other hormones, and in the fasted state a physiological IR occurs with increased concentrations of counter-regulatory hormones such as glucagon, and growth hormone. In some cases, excess secretion of these hormones may contribute to pathological IR (Wilcox, 2005), but in IR horses a compensatory downregulation of glucagon production is more common (Newkirk et al., 2018). The physiological states of pregnancy (Beythien et al., 2017) and stress (Andreazzi et al., 2011) also influence insulin sensitivity, but these relationships are complex and incompletely understood in the horse. Insulin resistance also occurs during systemic inflammatory response syndrome (SIRS) and has been induced by the infusion of lipopolysaccharide and by the overload of enteral carbohydrates in normal and EMS horses (Tadros et al., 2013, Toth et al., 2009). The direct inhibitory effect of inflammatory cytokines on intracellular signalling in response to insulin, or the indirect effect of counter-regulatory hormones such as cortisol are likely to cause this effect (Frank and Tadros, 2014).

Three pathophysiological processes have been implicated in the development of IR in people with metabolic syndrome: glucotoxicity, lipotoxicity and inflammation (Kim et al., 2006). The role of glucotoxicity appears to be less important in horses compared to humans, given the rarity of pancreatic insufficiency and hyperglycaemia. Persistent hyperinsulinaemia results in the development of IR in several rodent models (Shanik et al., 2008) and in horses, *ad lib* grazing (Fitzgerald et al., 2019b) or consumption of preserved forage (Lindase et al., 2018) results in a persistent, and in some horses marked, hyperinsulinaemia, which could drive the subsequent development of IR. This was supported by the observation that diets high in carbohydrate fed over 20 weeks resulted in the development of IR whereas isocaloric and presumably less insulinotropic diets high in fat, did not (Bamford et al., 2016a).

The accumulation of triglycerides in adipocytes during states of energy excess and obesity results in swelling of the cell and a state of maximum storage capacity. The diffusion distance for oxygen to the centre of the cell increases and this, accompanied by the impaired vasomotor control in adipose capillaries associated with IR, results in intracellular and

mitochondria stress and hypoxia within the adipocytes. Proinflammatory macrophage-infiltration and cytokine- and chemokine-expression then occurs (Goossens, 2008). The tissue also loses its ability to buffer lipid storage, and so ectopic lipid accumulation and lipotoxicity occur in other tissues such as the liver, causing organ dysfunction and exacerbating IR (Goossens, 2008). Obesity and increased concentrations of free fatty acids are a major contributory factor to IR and have a direct effect on vascular dysfunction in people (Kim et al., 2006). Increased triglyceride concentrations were measured in laminitis-prone ponies with IR (Bailey et al., 2008) and in ponies with hyperinsulinaemia (Morgan et al., 2014).

Evidence for an association between obesity and inflammation in horses remains limited and conflicting (Suagee et al., 2012), and is possibly confounded by the fact that acute hyperinsulinaemia has also been associated with increased pro-inflammatory cytokine expression (Suagee et al., 2011). Increased circulating tumour necrosis factor-α (TNF-α) concentration and increased interleukin-1 (IL-1) and TNF-α blood mRNA expression have both been associated with increased body condition score (BCS) and body fat composition in an age-dependent fashion in mares, supporting the theory that obesity induces a pro-inflammatory state in horses (Vick et al., 2007). These inflammatory cytokines can alter skeletal muscle response to insulin. For example, in humans, TNF-α has been shown to inhibit intracellular insulin signalling and IL-6 decreases insulin-dependent glucose uptake by skeletal muscle (Draznin, 2006). There is also evidence that different adipose deposits vary in cytokine profiles in horses, with neck crest fat having increased mRNA coding for interleukin-1β and IL-6 (Burns et al., 2010). However, in a different study, inflammatory cytokines measured systemically or in skeletal muscle were decreased in obese hyperinsulinaemic horses compared to controls (Banse et al., 2016). The link between obesity, inflammation and ID, and the order in which they occur, are incompletely understood in the horse and require further research.

The adipokines are a group of biologically active molecules secreted by adipose tissue with immune, cardiovascular and metabolic functions. Lipid dysregulation can occur as part of EMS (Durham et al., 2019), often associated with obesity, and disrupts normal adipokine secretion and function. The adipokines best characterised in the horse are leptin and adiponectin. Leptin regulates appetite, feed intake and therefore body weight via its target organ, the brain. Its concentration is closely related to adiposity (Buff et al., 2002). A state of leptin resistance results in hyperleptinaemia and inadequate modulation of appetite and

energy intake, resulting in obesity and IR (Caltabilota et al., 2010, Morgan et al., 2014). The relationship between insulin and leptin is complex, but insulin appears to be an important factor in stimulating leptin secretion from adipocytes and hyperleptinaemia and hyperinsulinaemia often coexist (Pleasant et al., 2013).

Adiponectin is also secreted from adipocytes, and its concentration is inversely related to adiposity and IR (Kearns et al., 2006, Wooldridge et al., 2012). It has insulin-sensitising and anti-inflammatory properties in humans (Whitehead et al., 2006). Low total adiponectin was recently associated with an increased risk of naturally occurring laminitis (Menzies-Gow et al., 2017), but high molecular weight adiponectin was not associated with occurrence of laminitis during an experimental high carbohydrate diet (Meier et al., 2020).

### *1.3.2 Basal hyperinsulinaemia*

Increased insulin concentration during a period of fasting or during consumption of low glycaemic feed is referred to as basal hyperinsulinaemia. It can result from any combination of a persistent stimulation of pancreatic β-cells, hyperresponsiveness of β-cells (Lindase et al., 2017), or reduced clearance of insulin (Toth et al., 2010), all of which can occur as a cause or a consequence of IR. In humans and horses, compensated IR is when the pancreas responds to peripheral IR by secreting more insulin, resulting in a compensatory hyperinsulinaemia and euglycaemia. If β-cells are unable to maintain sufficient insulin production then eventually uncompensated IR occurs (Treiber et al., 2005), resulting in hyperglycaemia and type-2 diabetes. Horses most commonly have compensated IR; pancreatic exhaustion and type-2 diabetes are rare in horses but a common long-term consequence in humans (Frank and Tadros, 2014). Pancreata from horses with chronic hyperinsulinaemia are larger and have an increased number of islets compared to those from normal horses (Morgan et al., 2020), indicating either an adaptive response by the pancreas to chronic stimulation, or that an increased number of islets is associated with the onset of hyperinsulinaemia.

In horses with experimentally induced or naturally occurring IR the rate of insulin clearance by the liver is reduced (Toth et al., 2010). Possible mechanisms including lipotoxicity and inflammation are discussed above. In addition, there is a reduction of insulin receptors at the hepatocyte cell surface during IR (Petersen and Shulman, 2018). The mechanism is not fully understood but the net effect of reduced insulin clearance is the promotion of hyperinsulinaemia.

### *1.3.3 Post-prandial hyperinsulinaemia*

Compensated IR is likely to be the main cause of postprandial hyperinsulinaemia, but recently a subgroup of animals that show postprandial hyperinsulinaemia without IR has been identified and a gastrointestinal aetiology has been proposed (de Laat et al., 2016). The existence of incretins has been established in the horse (Chameroy et al., 2010, Duhlmeier et al., 2001); in several studies GLP-1 and insulin concentrations have been correlated and an association with ID-status has been demonstrated (Bamford et al., 2016b, de Laat et al., 2016). There has been no definitive evidence linking abnormal incretin signalling and post prandial hyperinsulinaemia in the horse, however. Other neuroendocrine signals from the gastrointestinal tract which require further investigation for their postprandial insulinotropic effects include the cephalic phase of eating, signals in response to alterations in diet and the gut microbiota, and dietary endocrine disrupting chemicals.

Incretin-based therapies are commonly used to enhance insulin secretion in human type-2 diabetes, in particular GLP-1 analogues and DPP-4 antagonists (Nauck, 2016). Following preliminary *in vitro* studies (Kheder et al., 2018), GLP-1 antagonists merit further investigation in horses to help elucidate the role of GLP-1 in hyperinsulinaemia, and as a therapeutic strategy.

## 1.4 Epidemiology of Equine Metabolic Syndrome

There is minimal epidemiological data on EMS (Durham et al., 2019). Several studies have examined the prevalence of and risk factors for components of EMS, including laminitis (Pollard et al., 2019a, Pollard et al., 2019b, Wylie et al., 2013) and obesity (Thatcher et al., 2012, Wyse et al., 2008). Epidemiological studies on EMS defined by basal hyperinsulinaemia estimated a prevalence of 27% in ponies in Australia (Morgan et al., 2014), with risk factors identified including increasing age and supplementary feeding, and 18% of light-breed horses in Eastern USA (Pleasant et al., 2013). It is likely that both genetics and environment have key roles in the epidemiology of EMS, based on data from humans and laboratory animals (Pollex and Hegele, 2006). Obesity is a common problem in equine populations across the developing world with estimates of prevalence of 45% (BCS ≥ 5/6) in non-competition horses and ponies in Scotland (Wyse et al., 2008), and 72% (BCS ≥ 7/9) in ponies aged ≥7 years in Southeast England (Menzies-Gow et al., 2017).

### 1.4.1 Potential causal, or associated factors in EMS

#### 1.4.1.1 Genetics

It has been observed for many years that certain breeds are at increased risk of developing EMS (Geor, 2010, Jeffcott et al., 1986, Johnson, 2002). A clinical case series found that presentation for veterinary management of endocrinopathic laminitis was more likely in UK and Irish ponies compared to cold-blooded and Nordic breeds (Karikoski et al., 2011). Pedigree analysis in a closed herd of 160 pure- and crossbred Welsh and Dartmoor ponies was suggestive of a genetic basis for predisposition to pasture-associated laminitis (Treiber et al., 2006). Susceptible breeds often appear to be a 'thrifty' phenotype which was originally selected and bred to survive in regions with sparse, low non-structural carbohydrate (NSC) forage and a large seasonal variation in feed availability. It is likely that more complex genetic and epigenetic factors contribute, and confounding factors such as breed differences in management and athletic use must be recognised. Recent work has started to investigate heritability and genetic loci associated with EMS (Lewis et al., 2017, Norton et al., 2019a, Norton et al., 2019b), and this is an exciting area for further research.

#### 1.4.1.2 Signalment and history

It is recognised anecdotally that certain breeds (e.g., UK and Irish native ponies and cobs, Andalusians, Donkeys, Paso Finos, Morgans and Warmbloods) are at greater risk of developing ID and EMS. Early studies on a genetic basis for EMS have been limited to specific breeds (Lewis et al., 2017, Norton et al., 2019b), limiting application of this data to larger populations. Most horses with EMS are aged 5-15 years when veterinary examination is first requested for laminitis (Frank et al., 2010, Hart et al., 2016), but any age can be affected by EMS and more recently hyperinsulinaemia has been found to be associated with age (Hart et al., 2016, Morgan et al., 2014, Rapson et al., 2018). No association between EMS and sex has been detected, but hyperinsulinaemia and decreased insulin sensitivity have been demonstrated in mares in late pregnancy (Fowden et al., 1984)

An increased risk of laminitis is a defining feature of EMS, and so any history of laminitis, manifest either as detectable pain in the feet or morphological changes to the hoof (Karikoski et al., 2015) and without a compatible history for sepsis-related or weight-bearing laminitis, is likely to be associated with EMS.

#### *1.4.1.3* Management

Frequently EMS horses are described as 'easy keepers', with owners reporting that a small amount of feed is required to maintain body condition. In a recent study, ponies which had obesity induced over 20 weeks using a carbohydrate-rich diet developed ID, whereas ponies with obesity induced using an isocaloric fat-based diet did not develop ID (Bamford et al., 2016a). Horses on high-NSC diets are likely to be at increased risk of EMS compared to those on isocaloric fat-based diets. Overfeeding a high energy diet to Shetland ponies was shown to cause a short-term improvement in glucose metabolism, followed by significant hyperinsulinaemia (d' Fonseca et al., 2020).

Exercise has been shown to improve IR (Gordon et al., 2007a, Stewart-Hunt et al., 2006, Powell et al., 2002, Freestone et al., 1992), even in the absence of weight loss (Powell et al., 2002). The duration and intensity of exercise have varied considerably in these studies, however, and the durability of these improvements following cessation of exercise has been variable (de Graaf-Roelfsema et al., 2006). The exact intensity and duration of exercise required to have a beneficial effect on insulin sensitivity in not known and probably varies between individuals. Horses regularly performing high levels of exercise are at lower risk of EMS compared to more sedentary animals.

#### 1.4.1.4 Clinical examination

Certain phenotypic features are associated with EMS; however, these are not pathognomonic and may vary between breeds. Cardiovascular conditions are a common feature of IR in humans (Kim et al., 2006). In horses, hypertension (Bailey et al., 2008) and left ventricular hypertrophy (D'Fonseca et al., 2021) have been associated with ID, but other conditions seen in humans such as atherosclerosis and coronary artery disease do not feature. These cardiovascular parameters are unlikely to be useful for clinical assessment given inaccuracies of non-invasive blood pressure measurements in horses.

#### 1.4.1.5 Generalised or regional adiposity

Generalised obesity is normally defined as a BCS of ≥7/9 (Kohnke, 1992, Rendle et al., 2018) and has been associated with ID (Carter et al., 2009a, Fitzgerald et al., 2019a, Carter et al., 2009b, Pleasant et al., 2013) and an increased risk of laminitis (Carter et al., 2009c, Pollard et al., 2019b). Obesity has not been a consistent risk factor identified for the development of laminitis, however (Menzies-Gow et al., 2017). Normal, seasonal fluctuations in BCS occur in outdoor-living horses (Giles et al., 2014), and BCS should be slightly less at the end of the winter compared to the end the summer. Obese horses show less seasonal fluctuation in BCS

than normal horses (Giles et al., 2014), suggestive of adaptations of seasonal appetite and/or metabolism with the obese phenotype.

Regional adiposity has been assessed in several anatomical sites. A scoring system, termed the cresty neck score (CNS), for assessment of fat over the nuchal crest is commonly used and was first described by Carter (2009a). This has been associated with ID in a variety of breeds (Carter et al., 2009a, Fitzgerald et al., 2019a, Ragno et al., 2021). Carter et al. showed CNS to be a predictor of incipient laminitis in one closed herd of mixed breed ponies (Carter et al., 2009c), yet in another study on Welsh Ponies, CNS was found to be a poor predictor of ID during early weight loss (Dugdale et al., 2010).

#### *1.4.1.6* Evidence of current or previous laminitis

Laminitis is the most important disease expression associated with EMS. A subclinical form of laminitis with histological changes but no detectable foot pain can precede associated morphological changes visible on the hoof (prominent and divergent growth rings) by three months (Karikoski et al., 2015). Similarly, in a study of 38 client owned horses with confirmed PPID, 37% of animals were reported by owners to have a history of laminitis, but 75% were classified as having laminitis based on history, hoof morphology or radiography of the feet (Tadros et al., 2018). Recognition of foot pain in the horse can be challenging (Dutton et al., 2009), and these data indicate the importance of a clinical, and possibly radiographic, examination of the hoof when assessing laminitis.

## 1.5 Laboratory testing for EMS applicable to clinical practice

Insulin dysregulation and the associated hyperinsulinaemia is central to EMS and laminitis risk (Durham et al., 2019), and investigation of ID forms the mainstay of laboratory testing for EMS. Insulin resistance and hyperinsulinaemia can exist independently (de Laat et al., 2016), and in some individuals testing for both is required to definitively exclude ID. Laboratory testing for EMS most commonly involves measurement of serum or plasma insulin concentration.

### *1.5.1 Measurement of equine insulin concentration*

Numerous assays are available for the measurement of blood insulin concentration. Most assays used in equine clinical practice and research are designed for use in human medicine or human, rodent or porcine research, and there are no commercially available assays that use antibodies raised against equine insulin. Differences in amino acid sequence (Ho et al., 2011) mean that equine insulin is likely to have different binding affinities and cross-reactivity to the antibodies used in immunoassays compared to human, porcine and rodent insulin (Owen and Roberts, 2004). Furthermore, species-specific matrix interfering substances have the potential to add further inaccuracy with cross-species use of immunoassays (Park and Kricka, 2013). A commercial equine insulin standard (Shibayagi Co.[1]) for validation of assays (Banse et al., 2014) and setting of standard curves was available but has been discontinued, and there is no equine equivalent of the international standard for human insulin (Moore et al., 2019b). The gold standard assay is liquid chromatography – mass spectrometry (LC-MS), however it is not affordable or practical for clinical use and has only been used in one equine assay validation study (Tinworth et al., 2011).

The unit of measurement for insulin most used clinically and in research is µIU/mL, based on its activity. The SI unit is pmol/L, and mg/mL is also used in some publications. Using the standard conversion of 1 IU = 0.0347 mg for human insulin (Moore et al., 2019b), and with a molecular weight of human insulin of 5808Da, this means that 1 µIU/mL = 5.97 pmol/L.

Several insulin assays have been validated in horses as part of wider experimental studies (Knowles et al., 2016, Reimers et al., 1982) or as complete studies (Dunbar et al., 2016, Öberg et al., 2011, Warnken et al., 2016). An early comparison of assays in horses between three RIAs and one ELISA showed good correlation but poor agreement (McGowan et al., 2008), indicating that results from different assays should not be directly compared and emphasising the importance of assay-specific reference intervals. A larger study evaluated six commercial insulin assays, including two specifically marketed for horses, and then compared two (an RIA and an ELISA) against LC-MS gold standard measurements (Tinworth et al., 2011). The Coat-a-count RIA (Siemens[2]) performed adequately when charcoal-stripped serum was used as a diluent for parallelism, a finding supported in another study (Borer-Weir et al., 2012). It had moderate concordance ($\rho_c$ = 0.41) with LC-MS, but underestimated insulin concentration, with regression analysis showing RIA(µIU/mL) = 0.48 x (LC-MS) -7.7. At that time, the Coat-a Count RIA was commonly used in experimental and clinical studies and several recommended diagnostic cut-offs were established using this assay (Frank et al.,

2010, Treiber et al., 2006, Carter et al., 2009c). Unfortunately, the assay was discontinued in 2011. An ELISA assay optimised for equine use (Mercodia[3]) is available, which employs antibodies to porcine insulin and porcine standards. It has good precision, dilutional parallelism and recovery (Tinworth et al., 2011, Warnken et al., 2016, Öberg et al., 2011), but had very poor concordance to LC-MS (Tinworth et al., 2011). The Immulite chemiluminescent assay (Siemens[2]) is commonly used by commercial laboratories in the UK, as it offers rapid, automated processing of large number of samples. A comparison between the Immulite chemiluminescent and Coat-a-count RIA assays found poor agreement, although when 5 discordant samples were removed, bias and total error were within acceptable limits (Banse et al., 2014). Recommendations have been made for standardisation of human insulin assays (Staten et al., 2010), but no similar initiative has occurred in the veterinary field. Multiple assays with poor or unknown agreement continue to be used in experimental and clinical settings, making comparison of research findings and application to clinical practice challenging.

### *1.5.2 Basal tests*

#### 1.5.2.1 Insulin

Measurement of basal insulin concentration gives a non-specific indication of ID and has the advantage in clinical practice of only requiring a single visit and blood sample. The previously recommended prolonged fast prior to sampling and cut-off of 20 µIU/ml for diagnosis of EMS (Frank et al., 2010) results in a very low sensitivity (Dunbar et al., 2016, Lindåse et al., 2021, Olley et al., 2019) and is no longer recommended (Durham et al., 2019). Measurement of basal insulin whilst consuming low glycaemic forage or grazing, and a minimum of 4-5 h after any consumption of concentrates or grain is now recommended to increase sensitivity (Durham et al., 2019), and a reference interval of 2 - 21 µIU/mL has been recommended in horses consuming *ad lib* dry hay (Köller et al., 2016). Insulin response varies according to the NSC content of forage (Borgia et al., 2011, Lindase et al., 2018) and this needs to be considered when interpreting results. Recent work has shown that 75% of ponies with a 12 h fasted serum insulin >8.5 µIU/mL developed laminitis when subjected to a high NSC dietary challenge (Meier et al., 2018). An unfasted basal insulin of >21 µIU/mL had a sensitivity and specificity of 78% and 67%, respectively, for prediction of naturally occurring laminitis after 1 year (Menzies-Gow et al., 2017).

#### 1.5.2.2 Glucose

Horses with EMS and ID commonly have compensated IR, and hyperglycaemia is not a consistent finding. Fructosamine was shown to be increased in laminitic compared to normal horses (Knowles et al., 2012), but is rarely used diagnostically. Several proxies for IR using calculations of glucose and insulin concentrations have been described (Carter et al., 2009c, Treiber et al., 2005). In a recent study they had good repeatability but offered no diagnostic advantage over measurement of fasting insulin concentration (Lindåse et al., 2021).

#### 1.5.2.3 Leptin

Measurement of leptin concentration in horses is not commercially available in the UK, but it is available and used in the USA (Frank et al., 2020). Leptin has been identified as a risk factor for laminitis (Carter et al., 2009c), but it is unclear whether this association was independent of obesity, which was also identified as a risk factor. Leptin is strongly correlated with measures of adiposity (Gordon et al., 2007b, Kearns et al., 2006), but not IR (Bamford et al., 2016a). Based on current data it is indicated for diagnosis and monitoring of obesity, but not ID.

#### 1.5.2.4 Adiponectin

Adiponectin has insulin sensitising and anti-inflammatory functions and circulates in three main forms: trimers, low-molecular-weight hexamers or high-molecular-weight (HMW) multimers. The HMW form of adiponectin is negatively correlated with BCS and insulin concentration (Bamford et al., 2016a, Wooldridge et al., 2012) and in a longitudinal study total adiponectin was a predictor of laminitis in ponies (Menzies-Gow et al., 2017). In the period since these experimental studies took place the assays used have been changed or discontinued. Evaluation of alternative assays for total and HMW adiponectin showed reasonable performance of an immunoturbidimetric assay for measurement of total adiponectin, but inadequate performance of ELISAs for measurement of both HMW and total adiponectin (Menzies-Gow et al., 2018).

### *1.5.3 Dynamic tests for insulin regulation*

These tests measure response to orally or intravenously administered carbohydrate +/- exogenous insulin and are consider more sensitive for detection of ID compared to basal insulin. Oral dynamic tests measure the postprandial insulinaemic response to a measured amount of carbohydrate and are generally simpler to perform than intravenous tests.

Multiple enteric endocrine and metabolic factors will determine the insulinaemic response to oral carbohydrate, but the main advantage oral tests offer over intravenous tests is inclusion of the contribution of the enteroinsular axis to ID. In addition, as they reflect more closely the insulinaemic response to ingested carbohydrate, they have a greater relevance to pasture associated laminitis. Variability in rates of consumption, gastric emptying and intestinal absorption will affect the glycaemic response and assessment of IR (Kronfeld et al., 2005).

Several oral dynamic testing protocols have been described (Bertin and de Laat, 2017), but the oral glucose test (OGT) and oral sugar (corn syrup) test are currently recommended (Durham et al., 2019).

#### 1.5.3.1 Oral Glucose test (OGT)

In this test, a standard dose of glucose powder is mixed with a measured amount of water and low NSC fibre-based feed and fed as a single meal. After a defined period, usually 2 h, a blood sample is obtained and insulin, and sometimes glucose concentrations, are measured. A period of fasting before the meal is recommended to improve the horse's appetite and compliance with the test and reduce variability in gastric emptying and digestion. Weighing of any residual feed after 30-40 min aids with interpretation of results in case of incomplete consumption of the meal. Doses of glucose between 0.5 and 1 g/kg BW have been described (de Laat and Sillence, 2017, Durham et al., 2019, Meier et al., 2018). Reducing the dose of glucose from 1 to 0.75 g/kg BW in one study improved completion rate and, unexpectedly, resulted in a greater insulinaemic response. This suggests that intestinal absorption of glucose in 2 h might be saturated at doses ≥0.75g/kg (de Laat and Sillence, 2017).

The meal-based OGT has the benefit of avoiding the practical difficulties and stress for the horse associated with nasogastric intubation (Warnken et al., 2018) or oral syringing (Schuver et al., 2014). In humans and laboratory animals, a cephalic phase of insulin secretion occurs in response to visual and other stimuli associated with prehension and mastication of feed (Wiedemann et al., 2020). If a similar reflex occurs in the horse, then the OGT also has this advantage over non-meal-based tests. The disadvantage of relying on voluntary consumption of feed is loss of control over the rate and minimum amount consumed. This introduces variability to the rate of glucose absorption and insulinaemic response. Insulin concentration following a 0.75 g/kg BW OGT showed good repeatability (de Laat and Sillence, 2017), was associated with the development of laminitis during a high NSC diet challenge (Meier et al.,

2018) and was strongly correlated with insulinaemic response to grazing (Fitzgerald et al., 2019b)

Following a 1 g/kg BW OGT, an insulin concentration > 85 µIU/mL at 2 h is considered indicative of ID, measured using the Immulite 1000 analyser (Durham et al., 2019, Frank and Geor, 2014). Recent work from Australia quantifying the risk of developing laminitis during a high NSC feed challenge supported lower cut offs 2 h after a 1 g/kg OGT of 65 µIU/ml where resting basal insulin is < 8.5 µIU/ml or 50 µIU/ml based on 2 h insulin concentration only (Meier et al., 2018). Differences in the insulin assays used affect the translation of these results, however.

#### 1.5.3.2 Oral Sugar test (OST)

The Karo Light (not Karo Lite) corn syrup (ACH Food Companies[4]) used as the carbohydrate source in this test is available widely in the USA (Schuver et al., 2014), and can be purchased from online supermarkets in the UK. The syrup is administered by dosing syringe, offering the advantage over the OGT that the rate of consumption and total dose of carbohydrate is known and repeatable, producing a more consistently timed glucose peak (Schuver et al., 2014). Conventionally, a dose of 0.15ml of corn syrup/ kg BW has been recommended (Frank et al., 2020, Frank and Geor, 2014), however higher doses have resulted in higher and more discriminating insulin peaks (Jocelyn et al., 2018), and are now recommended (Durham et al., 2019). Fasting for 3-12 hours before the OST has been recommended (Bertin et al., 2016, Knowles et al., 2016), although it had no effect on dichotomous results in one study (Knowles et al., 2016). Timing of peak insulin concentration following administration of corn syrup might vary between horses and ponies (Smith et al., 2016), and recommendations for optimal sampling time have been between 30 and 90 min (Durham et al., 2019, Jocelyn et al., 2018, Schuver et al., 2014, Knowles et al., 2016). Obtaining two measurements 15-30 min apart has been recommended to increase the sensitivity of the test (Frank et al., 2020, Knowles et al., 2016).

#### 1.5.3.3 Intravenous Insulin tolerance test (ITT)

The ITT assesses tissue IR by measuring the glycaemic response to intravenous insulin administration (Caltabilota et al., 2010). A simplified, 2-step version of the full insulin response test has been recommended (Bertin and Sojka-Kritchevsky, 2013), which measures blood glucose before, and 30 min after IV injection of 100 mIU/kg of insulin. A ≥50% decrease in blood glucose concentration indicates normal insulin sensitivity. Results of the simplified

tests showed good agreement with the complete test. Hypoglycaemia can occur during this test, and if noted a starchy meal, hay or intravenous or oral glucose can be administered.

#### 1.5.3.4 Combined Intravenous Glucose- insulin tolerance test (CGIT)

The CGIT is a dynamic test which assesses but does not quantify insulin sensitivity. The standard CGIT protocol involves obtaining blood samples for measurement of glucose +/- insulin concentrations before and sequentially after intravenous boluses of 150mg/kg BW glucose and 0.1IU/kg BW insulin (Eiler et al., 2005). A shortened version of the test can be performed, with glucose and insulin being measured only at baseline, 45 min and 75 min (Durham et al., 2019, Morgan et al., 2015). Multiple parameters can be measured from the glucose and insulin response curves (Eiler et al., 2005), but essentially the rate at which glucose concentration returns to baseline reflects tissue sensitivity to the exogenous and any endogenous insulin, and the insulin concentrations at 45 and 75 min indicate endogenous insulin secretion in response to hyperglycaemia and/or rate of insulin clearance. Failure of glucose to return to baseline by 45 minutes or an insulin concentration > 100 µIU/mL at 45 min or > baseline at 75 min are indicators of IR (Eiler et al., 2005). In a group of clinically normal horses the repeatability of the insulin measurements was high, whereas for glucose measurements repeatability was low (Brojer et al., 2013). In the same paper, stress from transport affected the glucose, but not the insulin dynamics.

The CGIT is more time-consuming than oral tests and requires the horse to have a catheter placed the previous evening, making it less practical for use on the yard and in practice. It is useful for borderline cases, or those in which IR is suspected but which are negative with oral tests.

# 1.6 Aims and Objectives

The continued high incidence of endocrinopathic laminitis (Pollard et al., 2019a) has a detrimental effect on the welfare of the UK horse population. Equine metabolic syndrome (EMS), with the central feature of insulin dysregulation (ID), is a major cause of endocrinopathic laminitis (Durham et al., 2019). Like any medical diagnosis, identification of EMS and the associated increased risk for laminitis is done in 2 stages: First, horse- and management-level risk factors are used to identify individuals at increased risk. Management may be implemented at this point, based on financial constraints or an assessment that the horse is very likely to have EMS. Second, further laboratory testing is employed to confirm a diagnosis and to estimate the severity of ID and likelihood of it progressing to laminitis. Using prevalence and risk factors to estimate the pre-test probability of EMS in an individual enables a more accurate determination of the predictive value of the result and improves test interpretation. Starting at stage 2 and screening all horses with laboratory tests can be done, but may result in an increased proportion of false positive results. The risk of false positives is greatly reduced with high diagnostic test specificity or where the pre-test probability (based on prevalence and risk factors) before screening is high (Vetter et al., 2018). Therefore, an understanding of the prevalence and risk factors in a population will help direct decision making in diagnostic testing.

Compared to most diseases, the management of EMS, which consists principally of diet and exercise modification (Morgan et al., 2016a), is cheap and of low risk to the horse. It is a reasonable contention that the unnecessary treatment of some horses falsely diagnosed with EMS is acceptable if it minimises the number of horses with EMS that subsequently develop laminitis. However, dietary restriction, often recommended with increased stabling, can result in welfare implications for the horse and inconvenience for the owner (Furtado et al., 2021). Pharmacological options for management for EMS are increasing (Durham, 2017), and incur financial cost and risk adverse effects. Optimising the diagnostic accuracy of stages 1 and 2 should be prioritised.

The primary aims of the studies presented in this thesis were to estimate the prevalence of, and identify risk factors for, EMS in UK ponies and cobs, and to evaluate diagnostic testing for EMS.

The specific objectives of the studies presented in this thesis were:

1. To determine the effect of different processing and storage methods on measured insulin concentration in blood samples, relevant to transport to external laboratories (Chapter 2 and Supporting paper 1).
2. To evaluate the chemiluminescent insulin assay commonly used in diagnostic laboratories in the UK and compare it to the radioimmunoassay from which many diagnostic cut-offs have been derived (Chapters 3 and 7 and Supporting papers 2 and 4).
3. To compare the performance of existing diagnostic tests for insulin dysregulation in ponies (Chapters 4 and 7, Supporting paper 6)
4. To evaluate a novel oral carbohydrate test for EMS using a starch-based carbohydrate source which is a consistent composition and easily obtained by clients (Chapter 4).
5. To measure and compare glycaemic and insulinaemic responses to haylage, hay and soaked hay, and examine association with EMS status (Chapter 5 and Supporting papers 3 and 5).
6. To describe the glucagon-like peptide-1 response of ponies to preserved forages and oral carbohydrate tests and examine association with insulin response and EMS status (Chapter 6).
7. To estimate the prevalence of EMS in ponies and cobs in the UK (Chapter 7 and Supporting paper 4).
8. To identify signalment, management and phenotypic risk factors associated with hyperinsulinaemia and a positive EMS diagnosis following an oral carbohydrate test (Chapter 7 and Supporting papers 4 and 7).

## 1.7 Manufacturers' addresses

[1] Ishihara, Japan

[2] Siemens Healthcare, Camberley, Surrey, UK

[3] Uppsala, Sweden

[4] Cordova, Tennessee, USA

# Chapter 2: Serum insulin concentration in horses: effect of storage and handling

A modified version of this chapter has been published as a paper (short communication)

(See Supplementary information)

Carslake, H., Karikoski, N., Pinchbeck, G., McGowan, C. (2016)

Serum insulin concentration in horses: Effect of storage and handling

*The Veterinary Journal* 211, 94 – 96

## 2.1 Abstract

Serum insulin concentration is commonly measured during investigation of suspected endocrinopathic disease in horses, but immediate analysis is frequently unavailable. The aim of this study was to examine the effect of storing samples at room temperature for 72h as serum and as whole blood, compared to immediate separation and freezing.

Samples from 14 horses were evaluated. Correlation was excellent for all comparisons. Bland Altman plots revealed a negative bias (mean difference 2.16µIU/mL) in samples stored as whole blood compared to serum, but this difference was not considered clinically significant. At two commonly used diagnostic cut-offs there was no effect of storage on result. This study indicates that storage at room temperature for 72h, either as serum or whole blood, has minimal effect on measured serum insulin concentration in horses.

## 2.2 Introduction

Equine metabolic syndrome (EMS) and pituitary pars intermedia dysfunction (PPID) are commonly suspected in equine practice, especially in horses presenting with laminitis (Karikoski et al., 2011, McGowan et al., 2013b, Morgan et al., 2014). Both PPID and EMS have been associated with compensated insulin resistance or insulin dysregulation and measurement of serum insulin concentration basally, or following oral or intravenous glucose challenge remains an important part of the diagnosis and monitoring of affected horses (Durham et al., 2014, Morgan et al., 2015).

Following collection of the appropriate samples, most veterinary surgeons have to post them to external laboratories, in many cases unseparated due to having no immediate access to a centrifuge. Furthermore, samples taken on a Friday may take up to three days to reach a laboratory if routine postal services are used or laboratories are not staffed on the weekend. As such there is the potential for samples to be stored separated or unseparated for up to three days at room temperature.

The effect of storage and handling on a variety of clinicopathological parameters has been reported (Collicutt et al., 2015, Prutton et al., 2015, Rendle et al., 2009), but to the candidate's knowledge no published literature exists on the effect of storage prior to analysis on serum insulin concentration in horses.

The aims of this study were to examine the effect of storage at room temperature for 72 h, either as serum or whole blood, on serum insulin concentration. Based on the results of a pilot study we hypothesised that storage at room temperature for 72 h, either as serum or as whole blood would not significantly reduce serum insulin concentration compared to immediate separation and storage at -20 ˚C.

## 2.3 Materials and Methods

### *2.3.1 Sample acquisition and handling*

Blood samples from 14 horses (mean (range) age 14.4 (6-27) years), of mixed breeds, (7 mares, 6 geldings and 1 stallion) presented to the Helsinki University Equine Teaching Hospital, Finland for investigation of endocrine disease based on clinical signs of recurrent laminitis, PPID or phenotypic indicators of EMS between February and July 2008, were

included in the study. All blood samples were taken with informed owner consent for the purposes of clinical endocrine testing with aliquots used to perform the same test in triplicate using different storage conditions. Venous blood was collected from the jugular vein by single direct venipuncture into three identical evacuated tubes containing a clot activator (Vacuette, serum clot activator[1]) and allowed to clot at room temperature for 45 min. Samples CenRT and CenFr were immediately centrifuged at 2000 *g* for 10 min and the serum separated. Serum from sample CenRT was then maintained at room temperature (22 ˚C) in air-conditioning for 72 h. Serum from sample CenFr was immediately frozen at -20 °C. Sample RTCen was maintained at room temperature (22 °C) for 72 h, and then separated by centrifugation at 2000 *g* for 10 min. After 72 h, all samples were transferred to storage at -80 °C for a maximum of 7 months until analysis.

### *2.3.2 Sample Analysis*

All samples were packaged on ice and sent frozen to the same commercial laboratory by next-day delivery (Cambridge Specialist Laboratories[2]). Samples were analysed as a single batch using the DiaSorin S insulin RIA[3] validated for use in horses and previously described in horses (Karikoski et al., 2011).

### *2.3.3 Data Analysis*

Statistical analysis was performed using SPSS version 21[4], with 2-sided significance set at $P<0.05$. Comparisons were made between CenRT-CenFr, RTCen-CenFr and RTCen-CenRT. Agreement between groups was assessed using Bland Altman Plots, including analysis for proportional bias,and Linn's correlation coefficient with >0.99 indicating almost perfect strength of agreement, 0.95-0.99 substantial, 0.90-0.95 moderate and <0.9 poor. The mean value of the difference between the groups was compared to 0 using a 1-sample T test, to detect the presence of a fixed bias. Linear regression analysis was performed on the Bland Altman plot to detect proportional bias. Dichotomous outcomes were created using cut-off values for serum insulin concentration commonly used in clinical practice to diagnose EMS (30 µIU/mL for basal and 100 µIU/mL at 45 min during a combined glucose-insulin tolerance test (CGIT)). Agreement between groups was analysed using Kappa's measure of agreement.

### 2.3.4 Dates and location of the study and the candidate's role

Samples were collected between February and July 2008 in Helsinki, Finland and analysed in 2008 by a commercial laboratory in Cambridge, UK. Data were analysed in 2015. The candidate performed data analysis and prepared this chapter of the thesis.

## 2.4 Results

Measured insulin concentrations ranged from 6.6 – 310 µIU/mL (Table 1, Fig.1). Data analysis is summarised in Table 2. Bland Altman analysis (Fig. 2) showed a very mild bias for all three comparisons, however, the mean difference between CenRT and RTCen (2.16 µIU/mL) was significantly different from 0 (*P*=0.042). The 95% limits of agreement were narrowest for CenRT-RTCen and wider for the other comparisons, reaching -21.3 – 20.7 µIU/mL for CenFr–CenRT (Fig. 2). Linear regression of the Bland Altman plots showed significant proportional bias between RTCen and CenRT (*P*=0.004). Lin's concordance correlation coefficient showed almost perfect agreement between all three comparisons. For both 30 µIU/mL and 100 µIU/mL cut-offs Kappa's measure of agreement was perfect (Kappa statistic = 1).

**Table 2.1**

Summary of serum insulin concentration after three different storage and handling conditions in 14 horses.

| | **Serum Insulin (µIU/mL)** | | |
|---|---|---|---|
| | **CenFr** | **CenRT** | **RTCen** |
| Median | 49.5 | 50.0 | 47.0 |
| Interquartile range | 20.3 – 127.8 | 18.7 – 124.8 | 18.5 – 120.8 |
| Range | 7.2 - 279 | 7 - 310 | 6.6 - 299 |

*CenRT, centrifuged and serum maintained at 22°C for 72 h; CenFr centrifuged and serum immediately frozen at -20 °C; RTCen unseparated sample maintained at 22°C for 72 h before centrifugation*

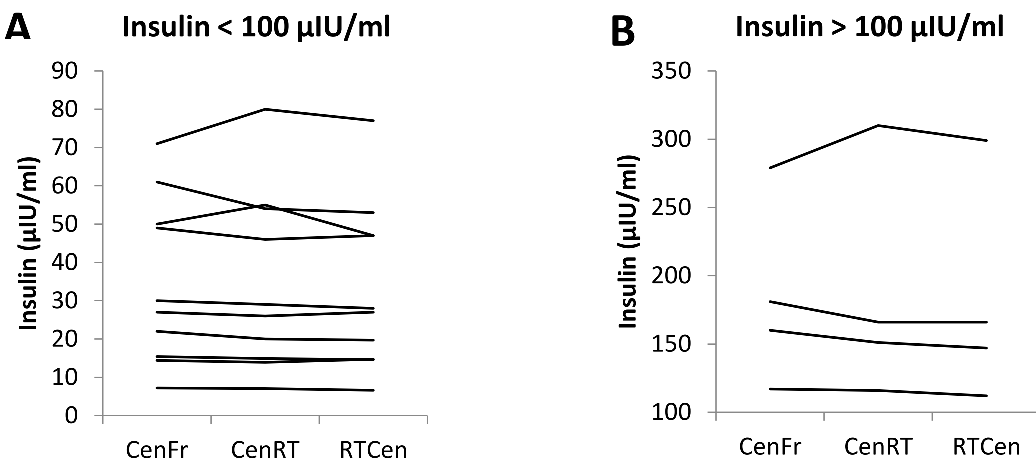



**Fig. 2.1.** Comparison of insulin concentrations in 14 horses after three different storage and handling conditions in low-medium (< 100 µIU/mL, A) and high (> 100 µIU/mL, B) ranges. *CenRT, centrifuged and serum maintained at 22°C for 72 h; CenFr centrifuged and serum immediately frozen at -20 °C; RTCen unseparated sample maintained at 22°C for 72 h before centrifugation*

**Table 2.2**

Comparison of serum insulin concentrations after three different storage and handling conditions, using Linn's concordance correlation coefficient, Bland Altman analysis and Kappa's measure of agreement for cut-offs of 30µIU/ml and 100µIU/ml.

| | | CenFr – RTCen | CenFr-CenRT - | CenRT - RTCen |
|---|---|---|---|---|
| Linn's Concordance Correlation coefficient (95% CI) | | 0.994 (0.983-0.998) | 0.992 (0.977 – 0.997) | 0.999 (0.997 – 0.999) |
| Bland Altman Analysis<br>Mean difference (µIU/mL) (95% LOA) | | 1.81 (-14.8 – 18.4) (*P*=0.42) | -0.34 (-21.3 – 20.7) (*P* =0.91) | 2.16* (-4.99 - 9.3) (*P* =0.04) |
| Regression $R^2$ | | 0.063 | 0.19 | 0.51 |
| Unstandardised Coefficient | | -0.03(*P* =0.39) | -0.06 (*P* =0.12) | 0.03 (*P* =0.004) |
| Kappa Measure of agreement value | 30µIU/mL | 1.0 | 1.0 | 1.0 |
| | 100 µIU/mL | 1.0 | 1.0 | 1.0 |

*(CenRT, centrifuged and serum maintained at 22°C for 72 h; CenFr centrifuged and serum immediately frozen at -20 °C; RTCen unseparated sample maintained at 22°C for 72 h before centrifugation. LOA, limits of agreement; * , mean difference significantly different to 0.*

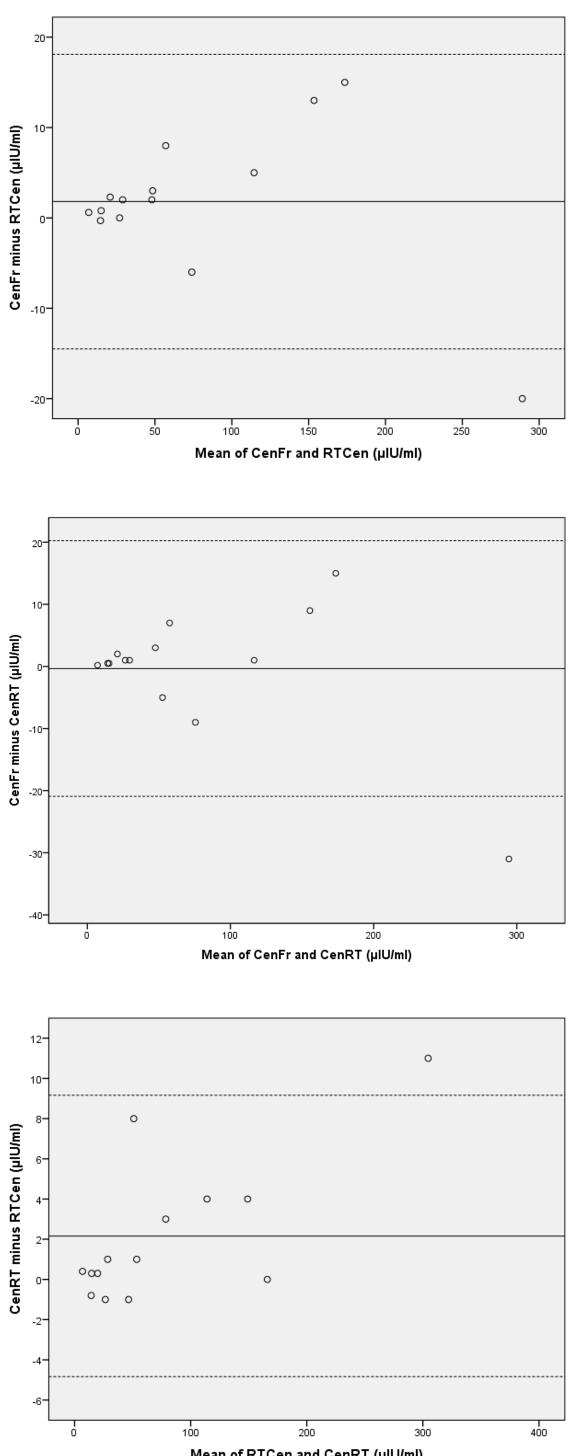



**Fig. 2.2.** Bland-Altman plots of the difference in serum insulin concentration against the mean for different storage and handling in 14 horses. The solid line is the average difference and the dotted lines represent 95% limits of agreement (values in Table 2). *CenRT, centrifuged and serum maintained at 22°C for 72 h; CenFr centrifuged and serum immediately frozen at -20 °C; RTCen unseparated sample maintained at 22°C for 72 h before centrifugation.*

## 2.5 Discussion

The agreement between samples in this study support that storage of serum or clotted blood at room temperature for 72 h has a clinically insignificant effect on serum insulin concentration, compared to immediate separation and freezing.

Although the 95% limits of agreement on Bland-Altman plots were quite wide, the mean differences were very small, and the larger variation was consistently found in horses with much higher insulin concentrations. The greatest variation in serum insulin was detected in one outlier with a high insulin concentration of 279-310 µIU/mL. Variation around the diagnostic cut-off values, which for this laboratory were 30 and 100 µIU/mL for basal and dynamic testing, respectively, were low and support the stability of serum insulin concentrations for clinical diagnostic purposes in horses. The mean difference between CenRT and RTcen was detected as a significant effect. This was associated with a mean difference of only 2.16 µIU/mL, which is unlikely to be of clinical significance. This difference supports that separation of serum helps prevent degradation of insulin and should be performed when possible.

Significant reductions in human insulin concentration have been demonstrated after storage for less than 24 h at room temperature (Oddoze et al., 2012). Given that human and horse insulin show substantial homology (Conlon, 2001) this apparent discrepancy is most likely explained by variation in study design (assay and statistical analysis), in the secondary, tertiary or quaternary structure of the different insulin molecules, or in insulin stability offered by human and equine serum.

Serum samples were stored at -80 °C for between one and seven months prior to analysis. Freezing of samples would not occur in clinical practice. In this study, the freeze-thaw cycle and the variable storage times might have influenced insulin concentrations.

Although the gold standard for any laboratory assay would be immediate analysis, this is frequently not available to veterinary surgeons in practice. Storage at room temperature for up to 72 h, either as serum or whole blood has minimal effect on serum insulin concentration compared to immediate separation and freezing. Immediate separation of serum is recommended to prevent a small reduction in measured insulin concentration.

## 2.6 Manufacturers' addresses

[1] Greiner Bio-One, Austria

[2] Cambridge, UK

[3] Saluggia, Italy

[4] IBM corp, USA

# Chapter 3: Evaluation of a chemiluminescent immunoassay for measurement of insulin concentration in equine serum

A modified version of this chapter has been published as a paper

(See Supplementary information)

Carslake, H.B., Pinchbeck, G. L., McGowan, C.M. (2017)

Evaluation of a Chemiluminescent Immunoassay for Measurement of Equine Insulin

*Journal of Veterinary Internal Medicine* 31 (2) 568 – 574

# 3.1 Abstract

Many diagnostic tests for insulin dysregulation use reference intervals established with an insulin radioimmunoassay (RIA) that is no longer available. A chemiluminescent immunoassay (CLIA) is commonly used for the measurement of serum insulin concentration in clinical practice but requires further validation, especially at clinically relevant reference intervals. The objectives of this study were to evaluate the CLIA for measurement of equine insulin and compare it to the previously validated, but now unavailable RIA. In this experimental study, equine serum samples (n=78) were obtained from clinical and experimental studies. Performance of the CLIA was evaluated using standard variables, including comparison to the RIA. Continuous and binary outcomes were analysed.

The CLIA showed good intra-assay (coefficient of variation (CV), 1.8-2.4%) and inter-assay (CV, 3-7.1%) precision. Acceptable recovery on dilution (100% ± 10%) was achieved only at dilutions <1:1. Recovery on addition was acceptable. Comparison of the CLIA and RIA showed strong positive correlation ($r$ = 0.91-0.98), with fixed and proportional bias. At 3 diagnostic cutoffs, sensitivity of CLIA compared to RIA ranged from 67-100%, and specificity from 96-100%. The CLIA is a highly repeatable assay which is suitable for within- and between-horse comparisons. Dilution of high concentration samples should be performed with charcoal stripped serum and at the lowest dilution factor possible. Agreement between the CLIA and RIA is acceptable. At concentrations commonly used for diagnosis of insulin dysregulation (≤100 µIU/ml) results from the CLIA tend to be lower and should be interpreted accordingly. Further standardization of equine insulin assays is required.

## 3.2 Introduction

Measurement of serum insulin concentration, both basal and after PO or IV glucose challenge, frequently is performed in equine clinical practice to determine the risk or cause of laminitis. Increased basal or dynamic insulin concentration is suggestive of insulin dysregulation, which has been shown to be central to the pathophysiology of endocrinopathic laminitis (Asplin et al., 2007, Jeffcott et al., 1986, Carter et al., 2009c, Karikoski et al., 2015, Treiber et al., 2006). Several insulin immunoassays are available commercially, most of which use antibodies against human or porcine insulin and none of which use antibodies specifically generated against equine insulin (Mercodia, 2016). Many of the reference ranges commonly used in clinical practice and experimental studies to diagnose insulin dysregulation (e.g., basal insulin >32 µIU/ml (Carter et al., 2009c) or insulin > 100 µIU/ml 45 min post-combined glucose-insulin test (Eiler et al., 2005)) are based on studies using a specific radioimmunoassay (RIA, Coat-a-Count[1]) that has been validated for use in horses (Tinworth et al., 2011), but is no longer commercially available. A chemiluminescent immunoassay (CLIA, Immulite 2000[2]) is commonly used in commercial laboratories for measurement of insulin concentration in clinical samples. One study compared the RIA and the CLIA and found poor agreement between the 2 assays, but when 5 discordant samples from a total of 40 were removed from the data set, bias and total error were within acceptable limits (Banse et al., 2014). Considering the common use of the CLIA in clinical practice and experimental studies, further evaluation of the assay is required. The purpose of our study was to evaluate the CLIA for measurement of equine serum insulin concentration and compare it to the previously validated but now unavailable RIA. We hypothesized that, at clinically relevant ranges, differences in serum insulin concentrations between the 2 assays would be clinically unimportant.

## 3.3 Materials and Methods

### 3.3.1 *Serum samples*

Excess equine serum from 3 sources was placed in aliquots and stored at -80°C: (1). Horses and ponies (n = 19, 8 mares and 11 geldings, mean (± s.d.) age 13.3 (± 4.3) years of mixed breeds) that were presented to the metabolic management clinic at the Philip Leverhulme Equine Hospital between October 2012 and March 2014 for suspected endocrinopathic

laminitis and that underwent a combined glucose-insulin tolerance test (CGIT) (Morgan et al., 2016a); (2). Two healthy ponies (a 4-year-old Welsh pony mare and a 5-year-old Australian pony gelding) maintained on a euglycaemic hyperinsulinaemic clamp during an experimental study performed in 2006 (Asplin et al., 2007); and (3). Ponies (n=12, 11 mares and 1 gelding, aged 9.1 ± 3.4 years and mixed UK native pony breeds) enrolled in a cross-over study examining insulinaemic responses to different feeds performed in 2014. Duration of storage varied according to collection date and the analysis being performed. Samples from sources 1 and 2 were analysed in 2014. Samples from source 3 were analysed from 2014 to 2016. Studies 1, 2 and 3 were approved by the University of Liverpool committee on research ethics, the Animal Ethics Committee of the University of Queensland, and a project license under the Animals (scientific procedures) Act 1986, respectively.

### *3.3.2 Precision: Intra- and Inter- assay coefficients of variation (CV)*

Intra-assay CV was calculated using 10 replicates of low (mean, 12.6 µIU/ml), medium (mean, 29.5 µIU/ml) and high (mean, 70.8 µIU/ml) insulin concentration samples from study 3 using the same CLIA cartridge.

Five control samples from study 3 representing a range of mean insulin concentrations (12.7 µIU/ml, 22.1 µIU/ml, 71.8 µIU/ml, 194.8 µIU/ml and 274.8 µIU/ml) were frozen in aliquots at -80 °C and used to determine interassay CV of the CLIA. Replicates were analysed on 6 different batches of reagents.

### *3.3.3 Dilutional parallelism and effect of diluent*

Four equine serum samples with initial, undiluted insulin concentrations of 83.2 µIU/ml, 150 µIU/ml, 217 µIU/ml and 281 µIU/ml from study 3 were diluted in ratios of sample:diluent of 2:1, 1:1, 1:2, 1:4 and 1:6, giving dilution factors of 0.33, 0.5, 0.67, 0.8 and 0.86, respectively. Samples were diluted with equine charcoal-stripped serum (CSS), the manufacturer-recommended, commercially-available assay diluent for the CLIA (Standard diluent, Siemens[3]), and 0.9% NaCl (Aquapharm[4]) solution to compare the effect of different diluents. Charcoal-stripped serum was prepared in advance based on a method previously described (Herbert et al., 1965) and the manufacturer's datasheet[5]. Dextran-coated charcoal (Sigma[5]) (0.02 g/ml) was added to equine serum, which was left on a laboratory rocker for 12 h at 4°C. The product then was centrifuged at 1000 *g* for 15 min, decanted, divided into aliquots and stored at -20°C until use. After thawing the serum was filtered through a 5 µm inline filter and analyzed to ensure that insulin concentration was zero. Linear regression was performed

to determine the intercept and slope for the lines for each diluent. Mean percentage recovery was calculated for each of the 3 diluents at each of the different dilution factors.

### *3.3.4 Recovery on addition*

Because equine insulin standard solutions are not commercially available, a human insulin standard solution (Immulite, Siemens[2]) was used with a mean measured concentration of 46.2 µIU/ml (range, 43-57 µIU/ml). Two different equine serum samples with low (3.2 µIU/ml) and medium (20.6 µIU/ml) measured insulin concentrations, and CSS (insulin concentration = 0 µIU/ml) were spiked with different volumes of the insulin standard. Recovery was calculated as a ratio of the measured increase in concentration to the predicted increase in concentration, and the mean value calculated.

### *3.3.5 Comparison between CLIA and RIA*

Serum samples (n=78) representing a wide range of insulin concentrations from sources 1 (n = 53 from 19 animals) and 2 (n = 25 from 2 animals) were thawed and assayed simultaneously using the CLIA and in duplicate using the RIA, according to the manufacturer's instructions. All assays were performed at the Institute of Veterinary Science's clinical pathology laboratory and by the same experienced laboratory technician. The maximum reportable concentrations for the RIA and CLIA were 350 µIU/ml and 300 µIU/ml, respectively; thus any samples with insulin concentration >300 µIU/ml on either assay (23/78 samples) were diluted 1:10 with CSS and repeated on both assays.

Samples below the lower detectable limit for each assay (2 µIU/ml for CLIA and 5 µIU/ml for RIA) were designated as having an insulin concentration of 0 µIU/ml, because this was considered to be a clinically unimportant difference. Based on how it was obtained, each sample was designated as either a basal insulin, 45 mins post-CGIT or hyperinsulinaemic clamp sample. Binary outcomes were created using cut-off values (20 µIU/ml and 32 µIU/ml for basal samples and 100 µIU/ml for 45 min post-CGIT) commonly used in clinical practice to diagnose insulin dysregulation. Hyperinsulinaemic clamp samples were excluded from the binary outcomes data.

### *3.3.6 Statistical Analysis*

Coefficient of variation (CV, %) was calculated as the ratio of the standard deviation to the mean multiplied by 100. An intra- or inter-assay CV ≤10% was considered acceptable. Dilutional parallelism was assessed using linear regression to determine the slope and

intercept of the line in comparison to 0 and 1, respectively. The percentage recovery on dilution and on addition were determined by calculating (measured concentration/expected concentration) ×100. Recovery of 100% +/- 10% was considered acceptable.

Comparison between the CLIA and RIA was performed using Deming's regression method (Deming, 1943), and the strength of the linear relationship using Spearman's rank correlation coefficient. Agreement between the 2 methods was performed using Bland-Altman analysis (Bland and Altman, 1986). These analyses were performed for all samples, and then also for samples with CLIA insulin concentrations <300 µIU/ml and <100 µIU/ml to provide more specific data for the most clinically relevant concentration ranges. Agreement between binary outcomes for RIA and CLIA was assessed using Cohen's kappa with κ value interpretation as <0.2 poor, 0.21-0.4 fair, 0.41-0.6 moderate, 0.61-0.8 good and 0.81-1 very good strength of agreement. Sensitivity and specificity of CLIA against RIA as the gold standard for each cut-off value was determined using 2 by 2 tables. Statistical analysis was performed using SPSS version 22 [6] and Medcalc version 16.4.3[7].

### *3.3.7 Dates and location of the study and the candidate's role*

Samples were collected from three experimental studies, one in 2006 (in Australia) and two in 2012 – 14 at the University of Liverpool. Laboratory work was performed in 2014 -16 at the University of Liverpool. The candidate was involved in the collection of some of the blood samples during the studies 2012-14. He contributed to study design and performed analysis of samples with assistance from laboratory technicians. The candidate performed data analysis and prepared this chapter of the thesis.

## 3.4 Results

### *3.4.1 Precision: Intra- and Inter- assay coefficients of variation*

Precision of the CLIA assay was excellent, with intra-assay CVs of 1.8%, 2.4% and 1.9% for low, medium and high insulin concentrations, respectively. Similarly, inter-assay CVs were all within acceptable limits, with 6.1%, 5.4%, 3.0%, 7.1% and 5.9% for samples with mean insulin concentrations of 12.7 µIU/ml, 22.1 µIU/ml, 71.8 µIU/ml, 194.8 µIU/ml and 274.8 µIU/ml, respectively.

### *3.4.2 Dilutional parallelism*

Dilutional parallelism with 3 different diluents of an equine serum sample with an initial insulin concentration of 217 µIU/ml is shown in Fig 1. Linear regression at this concentration indicated the best fit line as $y = 1.17x - 27.1$ ($r^2 = 0.99$, $P<0.001$) for CSS, $y = 0.96x - 26.7$ ($r^2 = 0.94$, $P = 0.007$) for standard diluent and $y = 1.18x - 39.1$ ($r^2 = 0.99$, $P = 0.001$) for 0.9% NaCl.

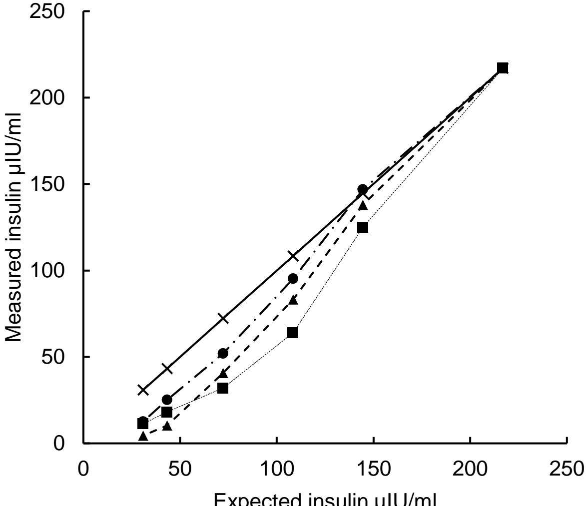



**Fig. 3.1**

Plot of measured against expected values for serum insulin after serial dilution of a high endogenous insulin concentration (217 µIU/ml) using charcoal stripped serum (circles) standard diluent (squares) and 0.9% saline solution (triangles). Crosses indicate measured = expected.

Mean percentage recovery from the 4 initial insulin concentrations was calculated for each of the diluents at different dilution factors and is shown in Fig 2. For all diluents, percentage recovery decreased as the dilution factor increased, and dilutional parallelism was not observed. Overall, mean percentage recovery was greater for CSS compared to standard diluent ($P < 0.0005$) and 0.9% NaCl ($P < 0.0005$). No significant difference in mean percentage recovery was detected between CSS and 0.9% NaCl at dilution factors 0.33 ($P = 0.49$) and 0.5 ($P = 0.29$). When using CSS as a diluent, the mean recovery at a dilution factor of 0.33 (2 parts sample:1 part CSS) was 90.9%, and at dilution factors ≥ 0.5 (1:1 sample:CSS), mean recoveries were ≤ 80.6%.

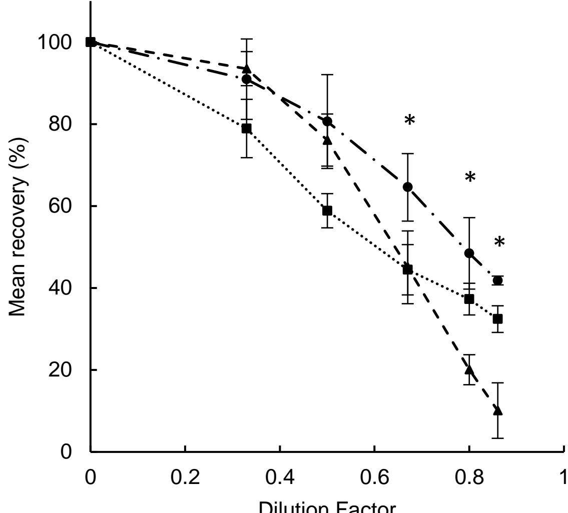



**Fig. 3.2**

Mean +/- standard deviation percentage recovery from four initial insulin concentrations plotted against dilution factor. Diluents were charcoal stripped serum (CSS) (circles) standard diluent (squares) and 0.9% saline solution (triangles).

* = significant difference ($P < 0.05$, 2-sided) in mean recovery between CSS and 0.9% saline solution. There was a significant difference in mean recovery between CSS and standard diluent at all dilution factors.

### *3.4.3 Recovery on Addition*

Recovery of the insulin standard is shown in Fig 3. The mean (95% confidence interval (CI)) percentage recoveries from the 3 different initial concentrations (0 µIU/ml, 3.2 µIU/ml and 20.6 µIU/ml) were all within the acceptable range, at 99.8% (CI, 92.1-107.5), 106.9% (CI, 98.0-115.8) and 100.1% (CI, 91.0-109), respectively.

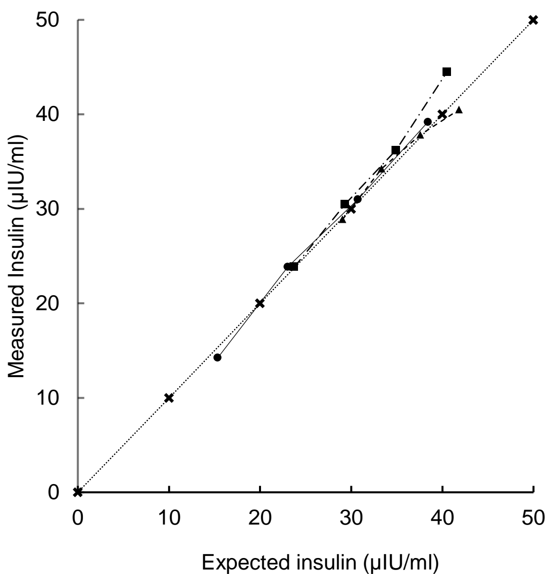
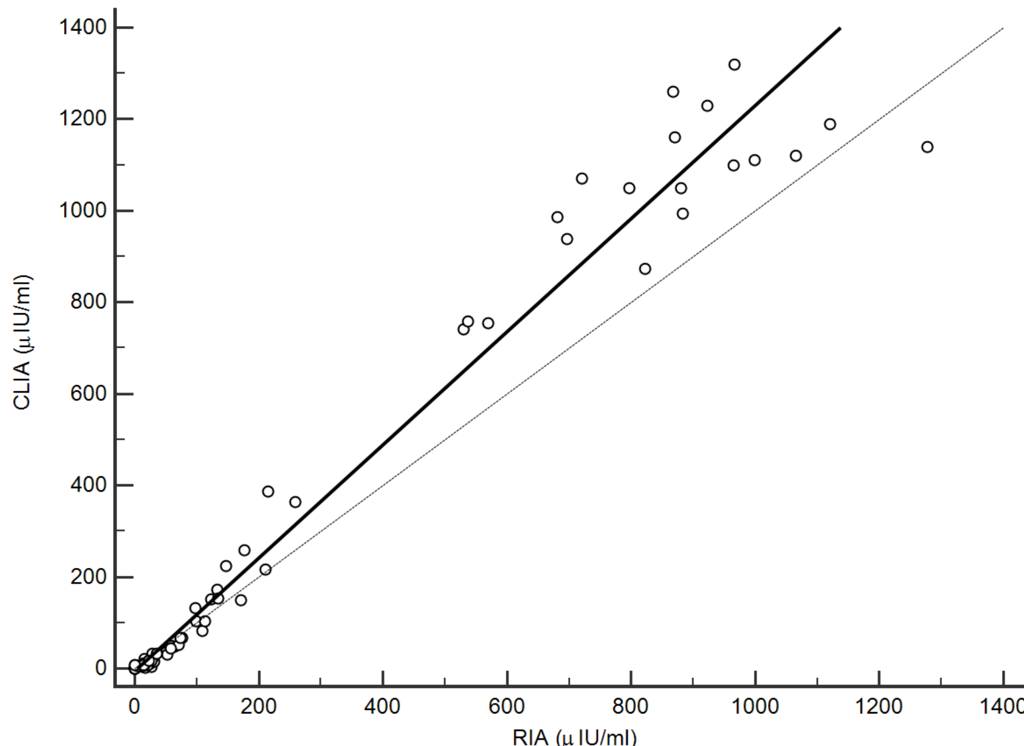



**Fig. 3.3**

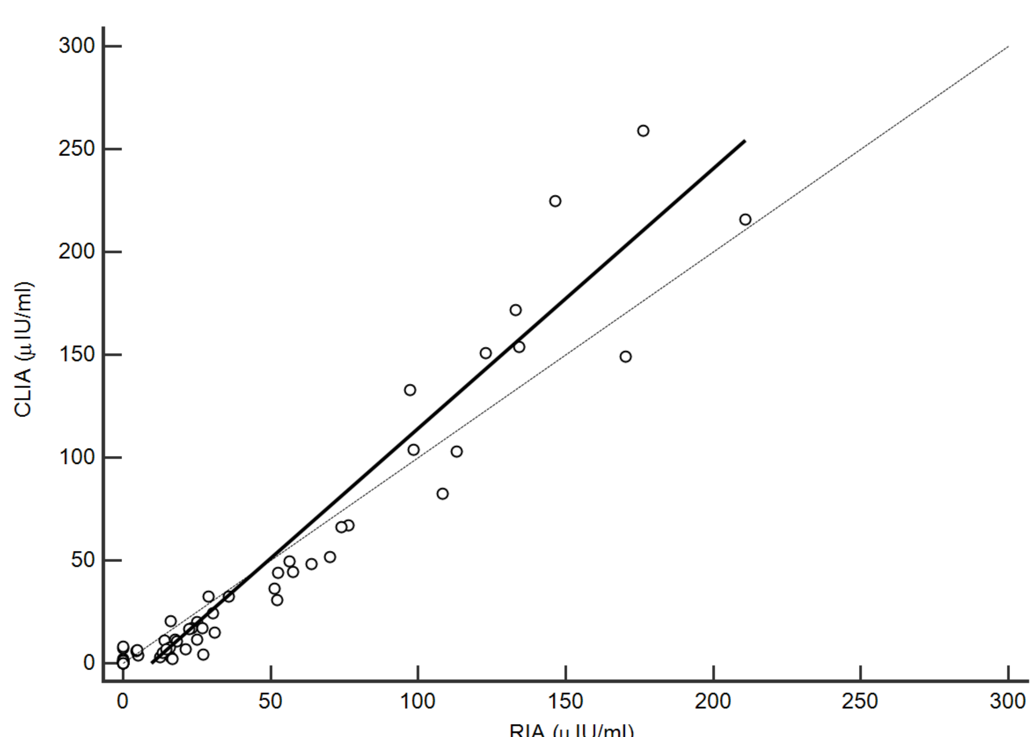
Recovery on addition of an insulin standard to three different low insulin concentration equine serum samples (Initial insulin concentration: circles = 0 µIU/ml, squares = low, triangles = medium). Measured concentration plotted against expected concentration. Dotted line = reference of measured = expected.

### *3.4.4 Comparison between CLIA and RIA*

Scatter plots of all CLIA concentrations, CLIA concentrations <300 µIU/ml and CLIA concentrations <100 µIU/ml against RIA concentrations with a line of best fit derived by Deming regression analysis and a reference y = x line are shown in Figs 4A, 4B and 4C, respectively. There was strong positive correlation ($r$ = 0.91–0.98, $P$ < 0.0005) in all 3 comparisons. For all CLIA concentrations (Fig 4A), the gradient (95% CI) of the best fit line was 1.24 (CI, 1.14 – 1.34), and the intercept (95% CI) was -7.98 (CI, -16.6 – 0.59). For CLIA concentrations <300 µIU/ml (Fig 4B), the gradient (95% CI) of the best fit line was 1.27 (CI, 1.04 – 1.49), and the intercept (95% CI) was -12.26 (CI, -19.35 - -5.16). For CLIA concentrations <100 µIU/ml (Fig 4C) the gradient (95% CI) of the best fit line was 0.84 (CI, 0.77 – 0.92), and the intercept (95% CI) was -2.4 (CI, -4.87 – 0.07).




B



C

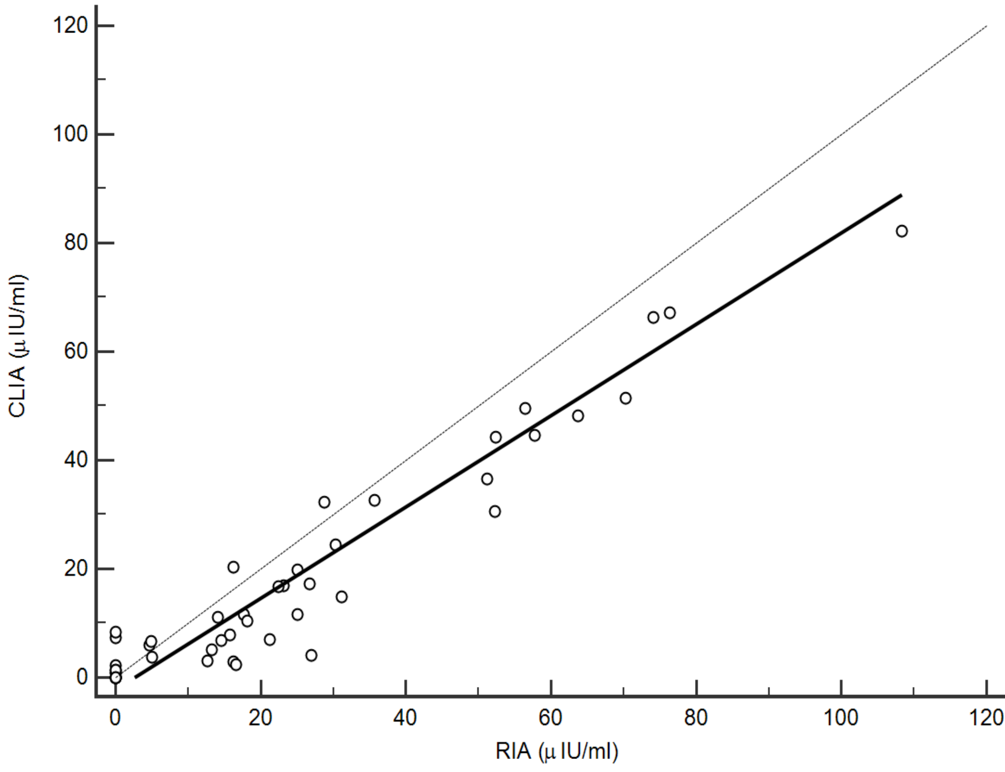
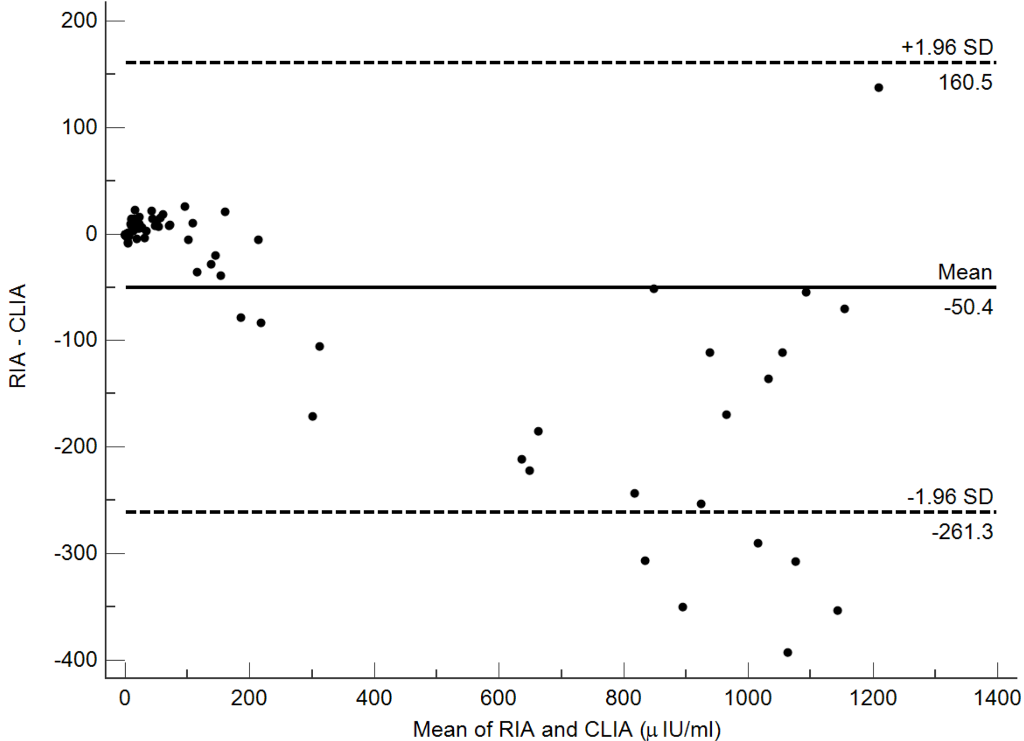


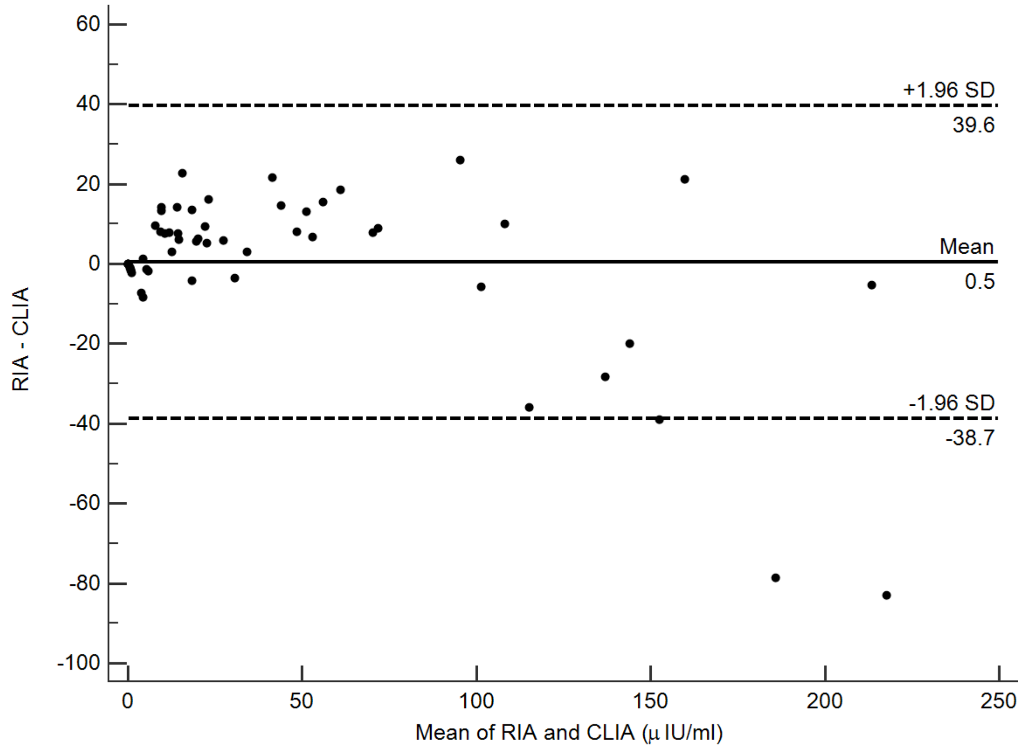

**Fig. 3.4**

Scatter plots of chemiluminescent immunoassay (CLIA) against radioimmunoassay (RIA) insulin concentrations, for (A) all samples (n=78), (B) samples with CLIA<300 µIU/ml (n=57) and (C) samples with CLIA <100 µIU/ml (n=47). All samples with an initial CLIA >300 µIU/ml were diluted 1:10 with charcoal stripped serum for both assays. The dotted line represents the line of best fit derived from Deming regression analysis and the solid line is a y=x reference.

A




B



C

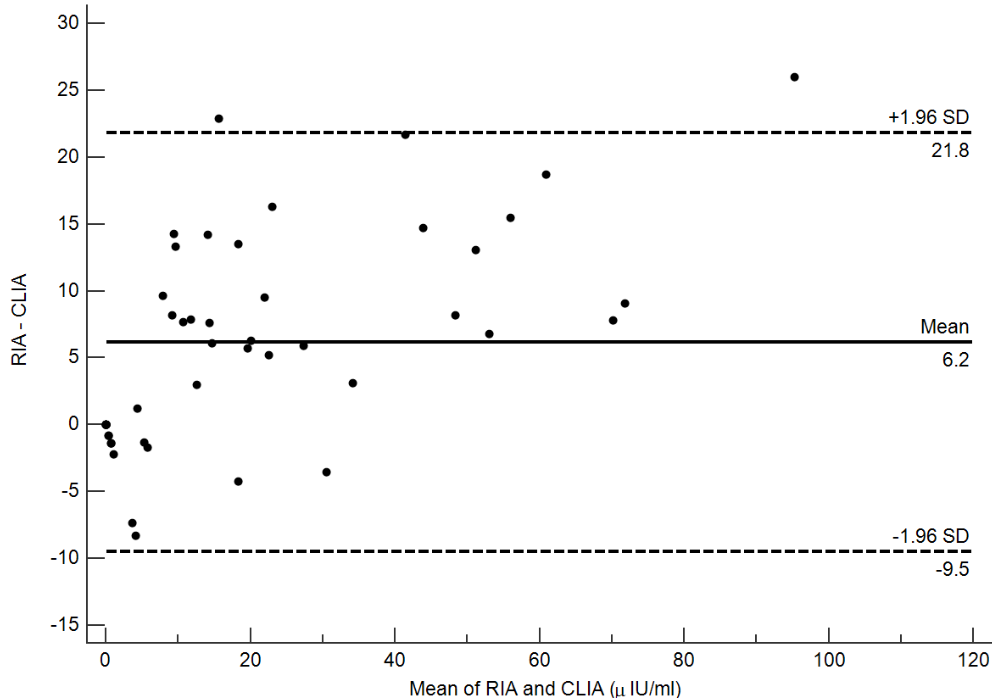



**Fig. 3.5** Bland-Altman plots of chemiluminescent immunoassay (CLIA) and radioimmunoassay (RIA) insulin results for (A) all samples (n=78), (B) samples with CLIA <300 µIU/ml (n=57) and (C) samples with CLIA <100 µIU/ml (n=47). Mean bias (solid line) and 95% limits of agreement (dotted line) are indicated and labelled.

A Bland Altman plot (Fig 5A) of all samples showed a fixed bias with CLIA concentrations a mean of 50.4 µIU/ml higher than RIA concentrations, and with 95% limits of agreement (LOA) of -261 – 160 µIU/ml. When only samples with CLIA < 300 µIU/ml were included (Fig 5B), the fixed bias (0.5 µIU/ml) was not significant, and 95% LOA were -38.7 – 39.6 µIU/ml. For samples <100 µIU/ml (Fig 5C), a fixed bias was present with RIA concentrations a mean of 6.2 µIU/ml higher than CLIA concentrations , with 95% LOA of -9.5 – 21.8 µIU/ml.

The kappa statistic of agreement between the RIA and CLIA for binary outcomes at diagnostic cut-off values for insulin of >20 µIU/ml was 0.66 (95% CI, 0.43 to 0.88; $P < 0.0005$), for >32 µIU/ml it was 0.94 (95% CI, 0.82 to 1; $P < 0.0005$), and for >100 µIU/ml it was 1.0 (95% CI, 1.0 to 1.0; $P<0.0005$).

Sensitivity, specificity and negative and positive predictive values of CLIA against RIA as the gold standard for the 3 diagnostic cut-offs are shown in Table 1.

**Table 3.1**

Sensitivity, specificity and positive- and negative predictive values for CLIA insulin concentrations against RIA concentrations, at three commonly used diagnostic cutoffs.

| Diagnostic cut off | Number of samples | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive value (%) |
|---|---|---|---|---|---|
| Basal Insulin > 20 µIU/ml | 34 | 67 | 96 | 92 | 81 |
| Basal Insulin > 32 µIU/ml | 34 | 100 | 97 | 91 | 100 |
| CGIT 45 min Insulin > 100 µIU/ml | 15 | 100 | 100 | 100 | 100 |

CGIT = combined glucose-insulin tolerance test

## 3.5 Discussion

Performance measures of the CLIA in our study indicate that it has high precision, both within and between runs and at a wide range of clinically relevant insulin concentrations. Accuracy of the assay is variable however with very good recovery on addition but acceptable recovery on dilution only when CSS or 0.9% saline is used and at low dilution factors. When compared to the previously validated RIA, serum insulin concentrations measured with the CLIA were strongly and positively correlated. Significant constant and proportional biases were detected, which varied according to insulin concentration. When binary outcomes at commonly used diagnostic cut-offs were compared, there were reasonable to good levels of agreement, sensitivity, specificity and predictive values.

The high intra- and inter-assay precision of the CLIA at clinically relevant insulin concentrations found in our study means that any differences detected over time or between samples are likely to be real rather than due to assay variability. A clinical example would be serial monitoring of basal insulin concentration before and after a period of dietary restriction. The CLIA compares favourably to the reported precision of other immunoassays for measurement of equine insulin, with the RIA, for example, having reported intra-assay CVs ranging from 4.4-10.7% (Borer-Weir et al., 2012, Öberg et al., 2011, Tinworth et al., 2011,

Tinworth et al., 2009) and other ELISAs having CVs as high as 19.9% (Borer-Weir et al., 2012). The automated processes used for the CLIA means that some of the imprecision that can be caused by operator error during manual assays (Tinworth et al., 2011) is avoided. All CLIA results in our study were obtained using a single analyser[2]. Other platforms in the same range of analysers exist, and although they use the same antibodies, reagents and standards, variability is possible among platforms and among individual analysers using the same platform. Ideally, normal reference intervals should be established for each individual analyser.

A diluent[3] consisting of concentrated insulin-free nonhuman serum matrix with preservative is supplied by the manufacturers of the CLIA intended for automated on-board (dilution factors specified by the operator) or manual dilution before loading of samples that exceed the reportable range of the assay (>300 µIU/ml). Although the CLIA still gives results for insulin concentration above this limit, it is recommended in the manufacturer's instructions that sample dilution be performed. To our knowledge no comparisons of diluents for the CLIA have been published. Our study showed that mean recovery at most dilution factors and initial concentrations was higher after dilution with CSS compared with the standard diluent or 0.9% NaCl. The exception was at dilution factors 0.33 and 0.5, where no significant difference in percentage recovery between CSS and 0.9% NaCl was detected. Two previous studies using the RIA showed similar results, where recovery of insulin was higher after dilution with CSS compared to phosphate-buffered saline, distilled water and the zero standard supplied (Borer-Weir et al., 2012, Tinworth et al., 2011). With all diluents in our study, percentage recovery of insulin decreased with increasing dilution, presumably due to interference with antibody binding or an alteration in the effective insulin concentration caused by substances in the diluent. Dilution factors > 0.33 decreased the percentage recovery to below 90%, so at high insulin concentrations the increased dilution required to bring the insulin concentration to within the reportable range might falsely decrease the final result. Hence, it is recommended that the lowest possible sample dilution be used to minimise this effect. At lower dilution factors, 0.9% NaCl offers advantages over CSS, such as ease of preparation and consistency among batches and laboratories, for no demonstrated difference in recovery. Given the recovery data above, a weakness in the design of our study was that all samples with an initial insulin concentration >300 µIU/ml were diluted 1:10 before being measured with CLIA and RIA. This is likely to have underestimated the insulin concentration in these samples more than if a lower dilution had been used.

The amino acid sequence of mammalian insulin is well conserved, with the equine insulin molecule differing by only 2 amino acids from human and 1 amino acid from porcine insulin (Ho et al., 2008). These differences result in alterations in secondary, tertiary and quaternary structure and antibody binding site conformation, leading to variable specificity of insulin immunoassays for different mammalian insulins. Although there is an ELISA that has been optimized for use in horses (Equine insulin ELISA, Mercodia AB[8]), no immunoassay currently available uses antibodies that have been raised specifically against equine insulin or equine insulin standards. An equine insulin standard (Shibayagi Co[9]) solution used in an earlier validation study (Banse et al., 2014) has been discontinued, meaning that human insulin standards had to be used for recovery on addition in our study. Recovery on addition was within acceptable limits (Fig 5), which contrasts with the poor recovery obtained with the equine insulin standard on the CLIA and RIA in the aforementioned study (Banse et al., 2014). This difference is most likely caused by the different types of insulin used, but the concentration of the standards was not confirmed with liquid chromatography-mass spectrometry (LC-MS) in either study and could have influenced results.

The lack of equine-specific standards or access to a reference assay makes assessing the accuracy of any equine insulin assay difficult. For the CLIA in our study, accuracy was determined by comparison to the RIA, because it has been used widely in experimental studies and showed the best performance in a study of 5 insulin assays for horses (Tinworth et al., 2011). The gold standard technique for insulin quantification is LC-MS (Rodriguez-Cabaleiro et al., 2007), but it was not used in our study due to lack of availability. To our knowledge, insulin LC-MS has been used to measure equine serum insulin concentration in only 1 study (Tinworth et al., 2011). In that study, insulin concentration determined by LC-MS had only moderate correlation with the RIA assay, with consistent underestimation of insulin concentration by the RIA. Standardisation of human insulin immunoassays with isotope dilution-liquid chromatography/tandem mass spectrometry resulted in successful reductions in total error (Rodriguez-Cabaleiro et al., 2007); a similar process could be applied to improve the accuracy of equine insulin assays.

There was a strong correlation between the RIA and CLIA in our study. There were small but statistically significant constant and proportional biases, however, which varied according to concentration. Results from the CLIA tended to be higher than those of the RIA for high

concentrations of insulin, whereas the opposite was the case for lower concentrations (<100 µIU/ml). The gradients and intercepts seen from Deming regression analysis line of best fit reflect this finding, and although most were significantly different from 1 and 0, respectively, the values were relatively small.

A limitation of our study was that most of the high insulin concentration (>300 µIU/ml) samples were from study 2 and most of the low samples were from study 1. The horses, diagnostic test performed, and sample storage time were different between the 2 groups of samples, and this difference might have influenced the relative performance of the CLIA and RIA. The euglycaemic hyperinsulinaemic clamp samples from study 2 (all of which had insulin concentrations >530 µIU/ml on RIA and > 742 µIU/ml on CLIA) will have contained largely recombinant human insulin, whereas the post-CGIT samples from study 1 are likely to have contained a mixture of human and endogenous insulin. As described above, antibody binding affinity can vary among heterologous insulin molecules and an assay comparison using samples with only endogenous insulin, for example after an PO sugar test, might yield different results. For all three sources of serum samples, multiple samples were obtained from individual horses, meaning they were not completely independent. This may have biased results. In addition, in our study any samples with an initial CLIA insulin concentration >300 µIU/ml were diluted with CSS before CLIA and RIA analysis, and this dilution might have affected final results differently for the RIA and CLIA.

Binary outcomes from the 2 assays at 3 different cut-offs demonstrated generally good performance of the CLIA compared to the RIA. The tendency for the CLIA to give lower results than the RIA at low concentrations is reflected in the lower sensitivity and negative predictive values seen for the 20 µIU/ml cut-off, but not the 32 µIU/ml cut-off, which had excellent sensitivity and specificity. Deming regression analysis of insulin concentrations <100 µIU/ml resulted in a best fit line gradient (95% CI) of 0.84 (CI, 0.77 – 0.92), meaning that when using RIA derived cut-offs, a CLIA result should be interpreted as having good specificity but lower sensitivity.

To summarise, the CLIA had good precision, recovery on addition and with CSS as the diluent and low dilution factors, adequate recovery on dilution. On this basis, the performance of the CLIA should be considered adequate for comparative measurements of equine insulin, for example monitoring response to dietary intervention, or comparing groups of horses in

experimental studies. The accuracy of the CLIA is difficult to assess with no specific reference standards or assays for equine insulin, and further research to establish standardisation and validation of assays is warranted. In comparison to the RIA, the CLIA had fixed and proportional bias. At the range of insulin concentrations most commonly used for diagnostic purposes (0-100 µIU/ml), there was reasonable agreement between the assays. Small proportional and fixed biases were detected which resulted in CLIA under-estimating the insulin concentration compared to RIA. When using diagnostic cut-offs for insulin dysregulation that have been determined using the RIA, CLIA results should be interpreted accordingly.

## 3.6 Manufacturers' addresses

[1] Coat-a-Count, Siemens, Camberley, Surrey, UK

[2] Siemens Healthcare, Camberley, Surrey, UK

[3] Siemens, Gwynedd, UK

[4] Animalcare, York, UK

[5] Dorset, UK

[6] New York, USA

[g] Ostend, Belgium

[h] Uppsala, Sweden

[I] Ishihara, Japan.

# Chapter 4: Comparison of five diagnostic tests for insulin dysregulation in ponies

## 4.1 Abstract

Clinical diagnosis of insulin dysregulation (ID) frequently involves oral tests in an attempt to capture horses with enteroinsular axis dysfunction. Tests using simple sugars may not reflect naturally ingested carbohydrates. This study aimed to compare one novel and two established oral carbohydrate tests with the intravenous combined glucose-insulin tolerance test (CGIT) and fasted insulin (FI) for diagnosis of ID in ponies. Twelve mixed-breed ponies maintained under identical management conditions were enrolled in a randomised crossover study. Following a period of fasting, ponies were administered an in-feed oral glucose test (OGT) (1g/kg glucose), oral sugar test (OST) (0.15ml/kg corn syrup), a proprietary cereal (WEET) (1g/kg non-structural carbohydrate), or a CGIT weekly. Glycaemic and insulinaemic responses were monitored for up to 5 hours. Analysis of continuous data and dichotomised results using standardised cut-offs was performed. Consumption of WEET was incomplete (33-73%) in 7 ponies; one was excluded (consumption 33%). Glucose and insulin responses were significantly greater to OGT compared to WEET and OST. Bivariate correlations between OGT, OST and WEET were all strong to very strong for area under the curve (AUC) and maximum insulin concentration, ($r = 0.85$-$0.94$, and $r = 0.87$-$0.92$, respectively, $P \leq 0.001$). AUC for glucose was significantly correlated between OGT and WEET ($r = 0.66$, $P = 0.03$) but not OST. Using conventional cut-offs for ID, dichotomous results showed substantial agreement between OGT and both WEET ($\kappa = 0.72$, $P = 0.03$) and OST ($\kappa = 0.67$, $P = 0.01$) and CGIT and both OST ($\kappa = 0.63$, $P = 0.03$) and OGT ($\kappa = 0.67$, $P = 0.01$), and no agreement between FI and all other tests ($\kappa = 0.17 – 0.42$, $P > 0.05$). For the diagnosis of ID compared to CGIT results, the sensitivity and specificity of OST were 75% and 88%, OGT were 100% and 75% and FI were 25% and 100%, respectively.

The three oral dynamic tests (OGT, OST and WEET) and CGIT resulted in reasonably comparable insulin responses and estimates of ID status. Fasting insulin lacks sensitivity for diagnosis of ID. The CGIT may have a lower sensitivity for diagnosis of ID than oral tests, and tests that include contribution of the enteroinsular axis are recommended. The WEET test offers a diagnostic feedstuff with a consistent NSC content, but palatability was poor. More work on development of an oral test using a more palatable feedstuff and appropriate cut-offs or diagnostic thresholds for tests of ID is warranted.

## 4.2 Introduction

Assessment of insulin dysregulation (ID) (Frank and Tadros, 2014) is required for a definitive diagnosis of equine metabolic syndrome and for the monitoring of management strategies (Morgan et al., 2016a). Several laboratory tests have been described for the assessment of ID (Bertin and de Laat, 2017), resulting in problems comparing clinical and experimental results.

Basal tests offer the convenience of a single blood sample without prior administration of glucose or insulin. Fasting insulin concentration (FI) has been recommended and is sometimes combined with other tests such as basal glucose and adipokines to provide proxies for insulin dysregulation (Treiber et al., 2005). Concerns over poor sensitivity of FI (Dunbar et al., 2016) have meant that measurement of insulin whilst on a low glycaemic feed is currently preferred (Durham et al., 2019), despite the loss of standardisation introduced by the variable insulinaemic effects of different forages (Borgia et al., 2011). A reappraisal of diagnostic cut-offs for FI has been advocated to improve diagnostic performance (Lindåse et al., 2021, Olley et al., 2019).

Intravenous dynamic tests offer a more direct assessment of tissue IR than basal tests. Their main advantage over oral tests is that the magnitude and timing of the blood glucose and/or insulin peak is known, making assessment of insulin resistance more accurate (Lindase et al., 2017). The intravenous combined glucose insulin test (CGIT) provides an indirect measure of insulin resistance and consists of an IV bolus of glucose immediately followed by IV insulin. The repeatability is good for the insulin curve, but poor for glucose dynamics (Brojer et al., 2013). It has been used in several clinical and experimental studies (Eiler et al., 2005, Morgan et al., 2016a, Ragno et al., 2021, Argo et al., 2012, McGowan et al., 2013a) and compared to most other IV dynamic tests is easy to perform. The main diagnostic outcomes are the duration of the positive phase of the glucose curve and insulin concentration at 45 min (Bertin and de Laat, 2017, Frank and Tadros, 2014) although experimentally, assessment of insulin concentration at other time points, especially 75 min, are used (Argo et al., 2012, Durham et al., 2019, McGowan et al., 2013a).

Oral dynamic tests are commonly recommended for clinical use (Durham et al., 2019). They are convenient to perform and, unlike intravenous tests, include the contribution of the gastrointestinal tract (the enteroinsular axis) to insulin secretion, estimated to be 25% in one study (de Laat et al., 2016). The oral glucose test (OGT) (Smith et al., 2016, Frank and Geor,

2014, Borer et al., 2012) assesses insulinaemic response following voluntary consumption of a meal consisting of glucose powder mixed with chaff. It predicted risk of developing laminitis in ponies fed a high non-structural carbohydrate diet in one study (Meier et al., 2018), and has shown reasonable repeatability (de Laat and Sillence, 2017). Disadvantages include variable palatability of larger doses of glucose (de Laat and Sillence, 2017) and poor availability of glucose powder for owners. The oral sugar test (OST) (Schuver et al., 2014) uses carbohydrate in the form of corn syrup administered by oral syringe. When compared with a dynamic IV test for IR, the OST lacked sensitivity (Dunbar et al., 2016), and higher doses of carbohydrate have been advocated (Jocelyn et al., 2018). It offers the advantage of a bolus of NSC at a single time point but requires compliance with syringing by both the horse and owner, and corn syrup may be less available to purchase in many countries. The OGT and OST were directly compared in eight insulin-sensitive horses and eight predominantly ID ponies, showing good agreement between the tests in most, but not all cases (Smith et al., 2016). A comparison with other tests was not performed.

An oral dynamic test using a commonly available and easily measured, palatable NSC source would offer advantages over the OST and OGT. A cereal-based feed test has been shown to produce hyperinsulinaemic responses that were predictive of subsequent laminitis (Meier et al., 2018) and may better represent natural feed stimulated insulinaemic responses to diet in horses (Meier et al., 2020).

To our knowledge, there has not been a direct four-way comparison of the four established diagnostic tests for ID (the OST, OGT, FI and an intravenous dynamic test) in ponies. Furthermore, these tests have not been directly compared with a cereal-based test. The objectives of this study were to: 1. Evaluate a cereal-based oral dynamic test for ID; 2. Compare the results between 4 established tests and a cereal-based test for ID; 3. Determine the level of agreement for binary results for a diagnosis of ID from these tests; 4. Using the CGIT as reference standard, establish a diagnostic cut off for the cereal-based test, and assess the sensitivity and specificity of the other tests.

## 4.3 Material and methods

The study was conducted under the Animals (Scientific Procedures) Act 1986 (project licence PPL 40/3715).

### *4.3.1 Animals*

Twelve adult ponies that had been maintained previously on a combination of preserved forages and pasture were purchased locally. To ensure that animals with a wide range of ID were included, ponies had been screened from 9 to 44 days before the start of the study using either a combined glucose-insulin tolerance test (CGIT, n = 10) or basal insulin (n = 2). Animals were excluded if they showed signs of pituitary pars intermedia dysfunction or clinical laminitis (grade > 0; Obel, 1948). Animals were confirmed to be free of other confounding disease by clinical examination, haematology, oral examination and, in animals >10 years of age, a basal adrenocorticotrophic hormone (ACTH) concentration <29 pg/mL. All ponies were dewormed on recruitment. Each week during the habituation period and throughout the study, bodyweight and girth and belly circumferences (cm) were measured, BCS (Kohnke, 1992) and cresty neck score (CNS) (Carter et al., 2009a), were estimated, and a clinical examination performed.

The ability of the novel test diet (WEET) to differentiate the horses into the same categories as the reference test (CGIT, high sensitivity and specificity determined by ROC analysis-derived cut off value) was considered as a preliminary validation of a prospective field test. Estimates for sample size for an acceptable sensitivity of 90% (95% CI 80—100%) for WEET to detect IR, and an anticipated prevalence of IR in this group of 75% was 12 animals.

### *4.3.2 Housing and feed*

Ponies were habituated to handling, stabling, feeding and weighing for 2 weeks prior to the start of the study. During this period and throughout the study, the ponies were individually stabled on wood shavings for 22 h/day and turned out with a closed grazing muzzle (Shires Equestrian[1]) into a 0.3 Ha paddock for free exercise for 2 h/day. Daily feed intake was standardised at 2% body weight (BW) fresh weight using same batch, dry meadow hay provided as two daily meals (0.5% BW at 12:00 after turnout and 1.5% BW at 17:00). A proprietary feed balancer (Lite Balancer, Spillers Feeds[2]) was provided (200 g/pony/day).

### *4.3.3 Study protocol*

This study was part of a larger, 7-way cross-over design where each pony received one of 7 interventions at 1-week intervals. Ponies received each intervention once, and the order was randomised (www.randomiser.com) for each pony. Each week, a catheter was placed in the jugular vein. That evening, hay provision was restricted (1% BW fed at 17:00) to ensure complete consumption by midnight. The following morning, each pony was randomly

allocated to one of the seven interventions, which were four dynamic diagnostic tests (OGT, OST, CGIT and a proprietary breakfast cereal (Weetabix[3], WEET)) and three forage meals (hay, soaked hay and haylage). Data from the four dynamic diagnostic tests is presented in this chapter.

### *4.3.4 Tests for insulin dysregulation*

Standard, previously described protocols were used for the OGT (Borer et al., 2012), OST (Schuver et al., 2014) and CGIT (Eiler et al., 2005). Briefly, for the OGT animals were given 1g/kg bwt glucose powder mixed with 1 g/kg bwt chaff-based feed (Happy Hoof, Spillers[2]) and 1 mL/kg bwt water. The OST consisted of 0.15ml/kg bwt corn syrup (Karo Light Syrup, ACH Food Companies[4]) syringed by mouth. For the WEET animals were given 1.46 g/kg bwt of Weetabix, equivalent to 1 g/kg bwt NSC, mixed with 2.2 ml/kg bwt water. The CGIT consisted of glucose (150 mg/kg) rapidly administered (< 1 min) as a 50% w/v solution, followed immediately by a bolus of neutral insulin (Humulin S, Eli Lilly[5]) (0.1 IU/kg), both administered intravenously. For the OGT and WEET time to consume the feed and the weight of any residual feed after 40 minutes was recorded. A baseline blood sample was obtained before all tests, and then 30, 60, 90, 120, 150, 180, 240 and 300 min following oral carbohydrate for the OGT, OST and WEET and 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135, 150 and 180 min after glucose and insulin administration for the CGIT.

### *4.3.5 Blood analysis*

Glucose was measured for all blood samples. Insulin was measured for all blood samples after OGT, OST and WEET, and 0, 25, 45, 60, 75, 90, 120, 150 and 180 min after CGIT. Blood for insulin analysis was collected into plain tubes and left at room temperature to clot for 1-2 h, after which it was centrifuged at 2000 *g* for 10 min and the serum separated. Insulin concentration was measured within 8 h of collection in duplicate by a chemiluminescent immunoassay (Immulite 2000, Siemens[6]) validated for use in horses (Chapter 3). Any samples with an insulin concentration greater than the upper range of the assay (>300 µIU/ml) were diluted 1:1 with activated charcoal (Sigma-Aldrich[7]) stripped equine serum and reanalysed. Blood for glucose concentration was collected into fluoride oxalate tubes and kept on ice until analysis within 8 h. Whole blood glucose was measured using a glucose oxidase enzymatic method (YSI 2300 STAT[8]).

### *4.3.6 Data Analysis*

Data from OGTs and WEETs were excluded if <50% of a meal was consumed after 40 min. Fasting insulin (FI) concentration was calculated as the mean concentration at t=0 min from the four dynamic tests. For OGT, OST and WEET, area under the curve for insulin ($AUC_i$) and glucose ($AUC_g$) were calculated using the trapezoid method with the x axis (*y*=0) as baseline. Maximum concentrations of insulin ($Cmax_i$) and for the CGIT, the duration (min) of the positive phase of the glucose curve relative to baseline ($CGITPP_g$) and insulin concentration at 45 min ($CGITins_{45}$) were derived from concentration/time curves. Normality of data was assessed using the Shapiro-Wilk test and when required, $log_{10}$ transformation performed to achieve normality before further analysis.

Comparisons of $AUC_i$, $AUC_g$ and $Cmax_i$ between OGT, OST and WEET were performed using repeated measures ANOVA with Greenhouse-Geisser correction and *post hoc* analysis with Bonferroni adjustment for all ponies, or Friedman analysis and *post hoc* Wilcoxon with Bonferroni adjustment for IR and IS subgroups. Comparison between IR and IS subgroups was performed using Mann Whitney U test. Pearson correlation coefficient (*r*) was calculated to examine the relationships between $AUC_i$, $AUC_g$ and $Cmax_i$ for OGT, OST and WEET, FI, $CGITins_{45}$ and $CGITPP_g$, with interpretation as follows: 0 - 0.1 negligible, 0.1 – 0.39 weak, 0.4 - 0.69 moderate, 0.7 – 0.89 strong and 0.9 – 1 very strong (Schober et al., 2018). Agreement for $AUC_i$ between OGT, OST and WEET was assessed using Bland Altman analysis (Bland and Altman, 1986).

For binary diagnostic cut offs, ID was defined as a FI >5.2µIU/mL (Olley et al., 2019), OGT insulin concentration at 120 mins ($ins_{120}$) >85µIU/mL, OST $ins_{60}$ or $ins_{90}$ > 35 µIU/mL (Durham et al., 2019), and CGIT $ins_{45}$ >82 µIU/mL or a $PP_g$ > 45min (Eiler et al., 2005). Where required, diagnostic cut-offs using the Immulite 2000 assay in this study were calculated from the original values from the Coat-a-Count RIA assay using the formula y = 0.84x -2.4 (Chapter 3).

Median time to maximum concentration of insulin ($Tmax_i$) was calculated for WEET. Using the CGIT result as the reference standard for insulin resistance (IR), receiver-operator curve analysis was used to calculate the optimal cut-off for WEET at $Tmax_i$ as a diagnostic test for IR. Agreement between dichotomised ID status from the different tests was assessed using Cohen's Kappa, with scores interpreted as follows: <0 none, 0-0.2 slight, 0.2-0.4 fair, 0.4-0.6 moderate, 0.6-0.8 substantial and 0.8-1 almost perfect (Landis and Koch, 1977). Sensitivity and specificity of OGT, OST and FI for diagnosis of IR were calculated using CGIT as the gold standard. Sensitivity and specificity of WEET was not included as the cut off was derived from

the same set of CGIT results. Statistical analysis was performed using commercial software (SPSS version 27, (IBM[9]) and MedCalc version 20.014[10]), with significance (2-sided) set at $P < 0.05$.

### *4.3.7 Dates and location of the study and the candidate's role*

The cross over study, including analysis of samples took place in 2014 at the University of Liverpool. The candidate contributed to study design and organised and performed data collection and sample analysis with assistance from technicians. He performed data analysis and prepared this chapter of the thesis.

# 4.4 Results

### *4.4.1 Animals*

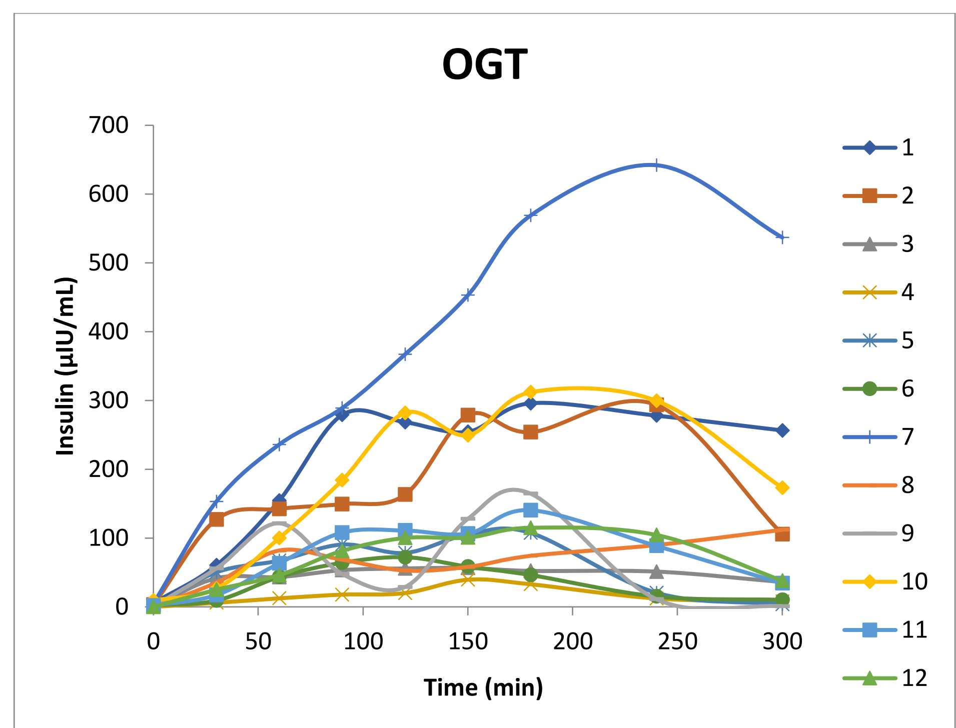
All ponies were mixed, native British breeds. There were 11 mares and one gelding, aged (mean ± s.d.) 9.1 ± 3.4 years and with a body weight of 280 ± 49 kg. The median BCS was 7.2 (IQR 6.0 – 7.7) out of 9, with a range of 4.2–8.5. There was no significant difference in mean body weight, girth or belly circumference, CNS or BCS at the time the different tests were performed, or between IR and IS groups. Over the 6–week study period, mean body weight increased from 278 ± 51.2 kg to 284 ± 52.6 kg ($P$ = 0.001).

The OGT meal was entirely consumed by 9/12 ponies, taking (mean ± s.d.) 23.4 ± 10.7 min. Ponies 1, 9 and 11 consumed 94%, 85% and 93% of the OGT meal respectively. The WEET meal was entirely consumed by 5 ponies, taking 21.8 ± 13.3 min. Ponies 1, 4, 5, 6, 10, 11, and 12, consumed 66%, 66%, 67%, 73%, 56%, 71% and 33% of the WEET meal respectively, with pony 12 excluded from further analysis. All tests were well tolerated with no adverse effects seen.

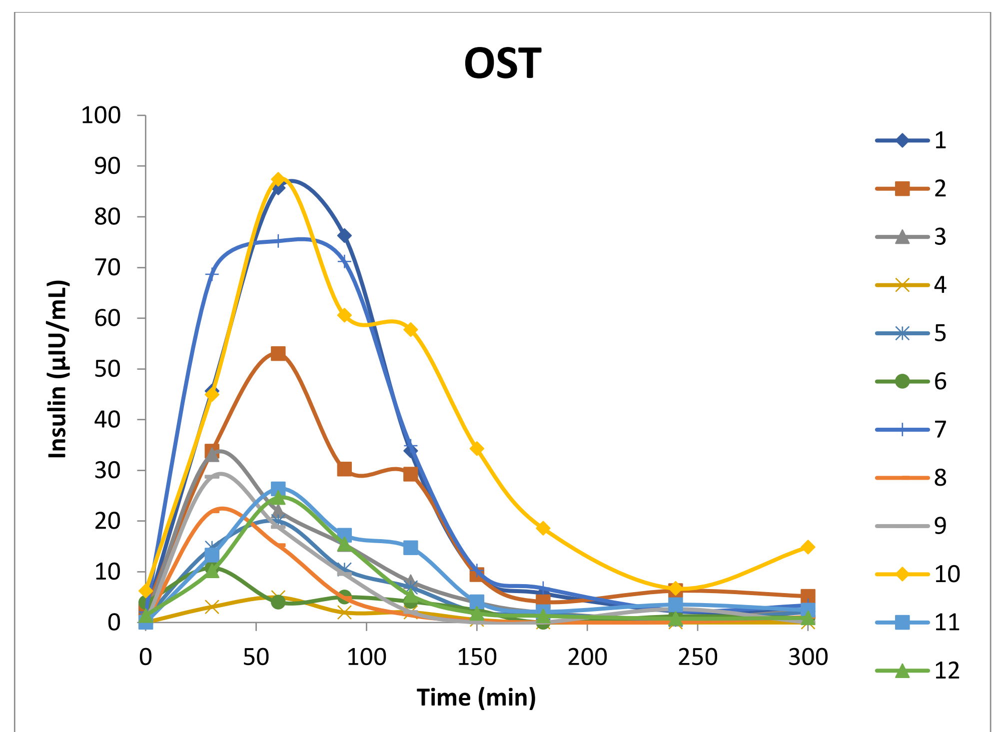
**Table 4.1:** Results (median (IQR)) for area under the curve for insulin ($AUC_i$) and glucose ($AUC_g$), and maximum concentrations of insulin ($Cmax_i$) following oral glucose test (OGT), oral sugar test (OST) and cereal test (WEET), positive phase of the glucose curve ($CGITPP_g$) and insulin concentration at 45 min ($CGITins_{45}$) after a CGIT, and fasting insulin (FI) in 12 ponies. $^{a\text{-}g}$ Values in the same column with the same superscript letter are significantly different ($P$ < 0.05, 2-sided). * significant differences ($P$ < 0.05, 2-sided) between insulin sensitive and insulin resistant ponies.

| | | All Ponies (n=12) | Insulin Sensitive (n=8) | Insulin Resistant (n=4) |
|---|---|---|---|---|
| **$AUC_g$ (mmol min/l)** | OGT | 1488$^{a}$ (1362 - 1856) | 1407$^{d}$* (`344 - 1491) | 1892* (1753 - 2028) |
| **$AUC_g$ (mmol min/l)** | OST | 1105$^{a}$ (1073 - 1153) | 1105$^{d}$ (1080 - 1135) | 1132 (1061 - 1196) |
| **$AUC_g$ (mmol min/l)** | WEET | 1247$^{a}$ (1165 - 1576) | 1198* (1105 - 1301) | 1615* (n/a) |
| **$AUC_i$ (µIU min/mL)** | OGT | 22100$^{b}$ (15026 - 61467) | 18760$^{e}$* (11580 - 23906) | 65441* (33046 - 111007) |
| **$AUC_i$ (µIU min/mL)** | OST | 2289$^{b}$ (1463 - 7357) | 1864$^{e}$* (1047 - 2677) | 8192* (3406 - 9836) |
| **$AUC_i$ (µIU min/mL)** | WEET | 12172$^{b}$ (4891 - 54068) | 7650$^{e}$* (3113 - 13630) | 60409* (n/a) |
| **$Cmax_i$ (µIU/mL)** | OGT | 127.8$^{c}$ (81.1 – 296.4) | 109.8$^{fg}$* (60.5 – 158.5) | 304* (160 - 560) |
| **$Cmax_i$ (µIU/mL)** | OST | 27.5$^{c}$ (20.4 – 69.7) | 24.1$^{f}$* (13.1 - 32) | 80.5* (37.3 – 86.9) |
| **$Cmax_i$ (µIU/mL)** | WEET | 75.4$^{c}$ (26.6 – 294.5) | 47.0$^{g}$* (18.8 – 91.5) | 296.5* (n/a) |
| **FI (µIU/mL)** | | 2.2 (0.67 – 3.75) | 1.3 (0.4 – 2.7) | 3.7 (1.4 – 8.7) |
| **CGITPPg (min)** | | 29.5 (20.5 – 42.8) | 24* (18.5 – 29.8) | 44.5* (38.8 – 53.2) |
| **$CGITins_{45}$ (µIU/mL)** | | 48.4 (34.4 – 97.5) | 44.1* (29.0 – 49.6) | 112.5* (65.2 – 118.4) |

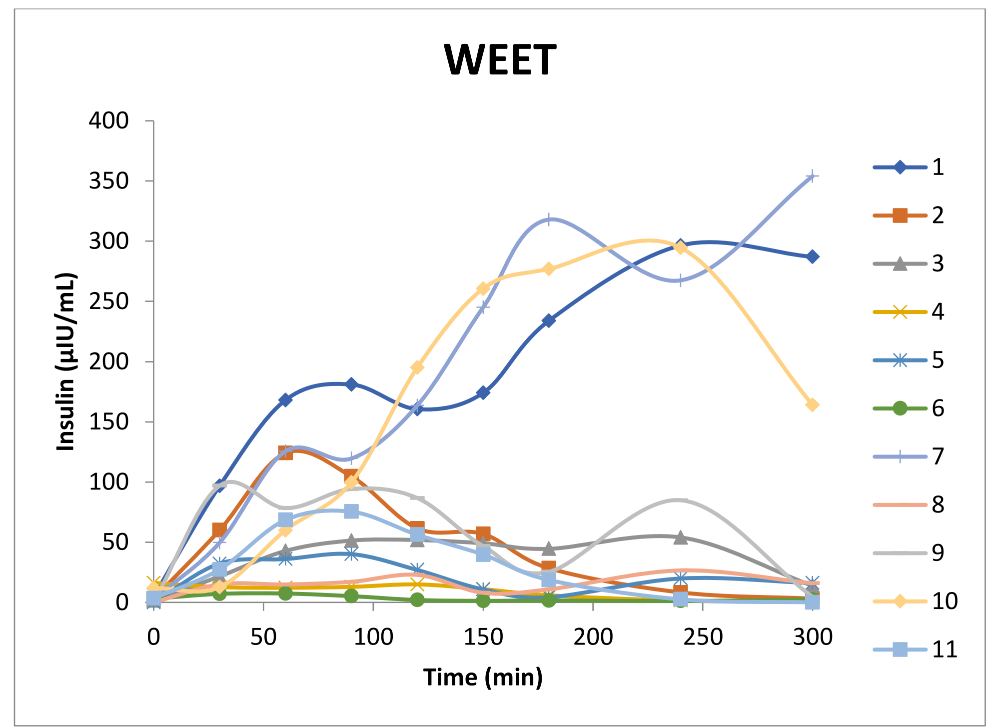
a)




b)



c)




d)

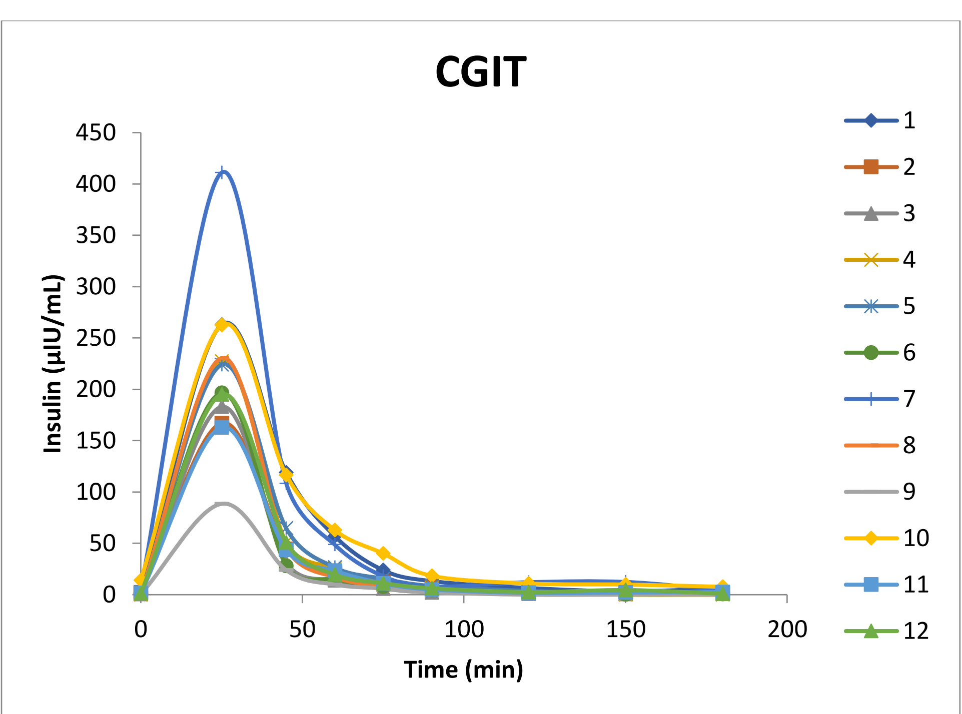
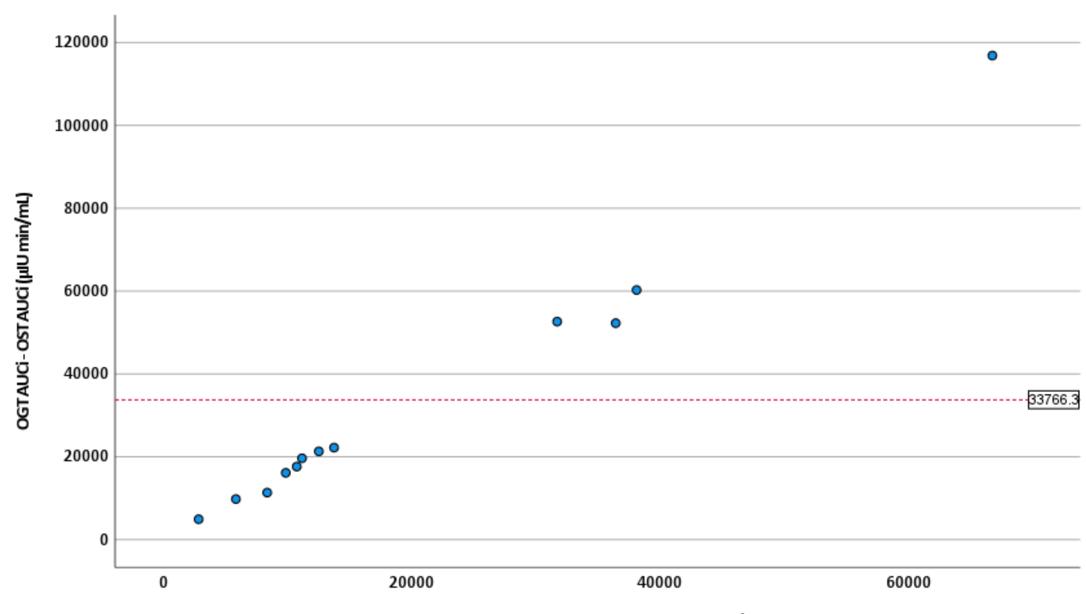



**Fig. 4.1:** Serum insulin concentration (µIU/mL) following a) oral glucose test (OGT), b) oral sugar test (OST), c) cereal test (WEET) and d) combined glucose insulin test (CGIT) in 12 ponies (numbered 1-12). Pony 12 was excluded from WEET analysis as <50% of the meal was consumed.

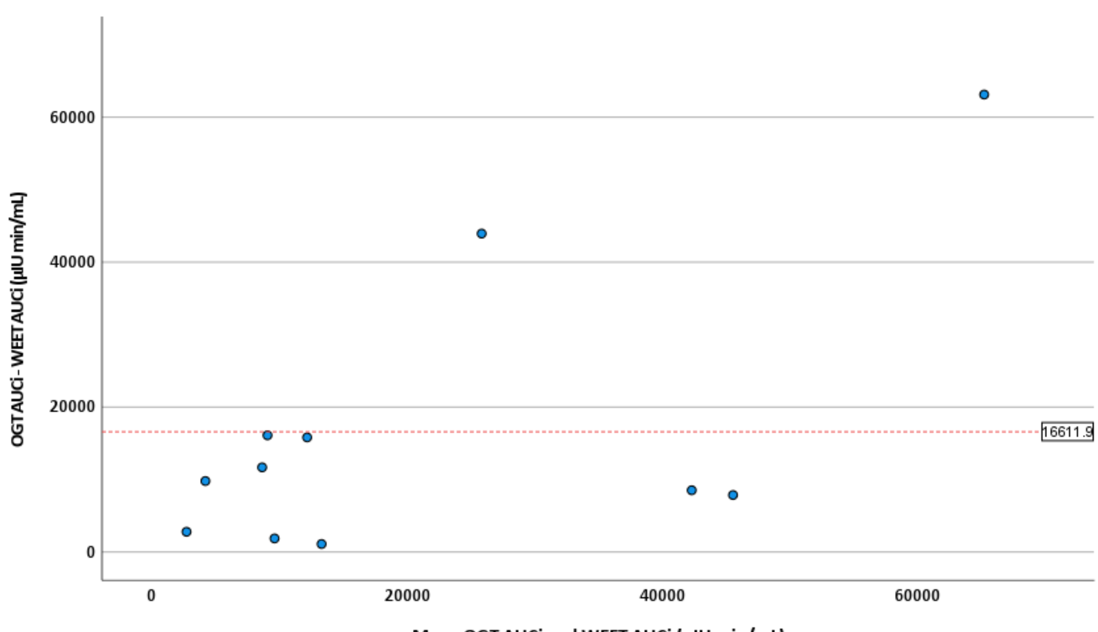
**Table 4.2:** Pearson correlation coefficient (*r*) with upper and lower 95% confidence interval limits (95% CI) for relationships between area under the curve for insulin ($AUC_i$) and glucose ($AUC_g$), and maximum concentrations of insulin ($Cmax_i$) following oral glucose test (OGT), oral sugar test (OST) and cereal test (WEET), positive phase of the glucose curve ($CGITPP_g$) and insulin concentration at 45 min ($CGITins_{45}$) after a CGIT, and fasting insulin (FI) in 12 ponies, after $log_{10}$ transformation for non-parametric data. ** *P* <0.01, * *P* <0.05

| | | $Log_{10}$ OGT $AUC_i$ | $Log_{10}$ OST $AUC_i$ | $Log_{10}$ WEET $AUC_i$ | OGT $AUC_g$ | OST $AUC_g$ | WEET $AUC_g$ | $Log_{10}$ OGT $Cmax_i$ | $Log_{10}$ OST $Cmax_i$ | $Log_{10}$ WEET $Cmax_i$ | $Log_{10}$ FI | $Log_{10}$ $CGITins_{45}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $Log_{10}$ OST $AUC_i$ | *r* | .940** | | | | | | | | | | |
| | 95% CI | 0.80, 0.98 | | | | | | | | | | |
| $Log_{10}$ WEET $AUC_i$ | *r* | .849** | .891** | | | | | | | | | |
| | 95% CI | 0.51, 0.96 | 0.63, 0.97 | | | | | | | | | |
| OGT $AUC_g$ | *r* | .783** | .690* | .704* | | | | | | | | |
| | 95% CI | 0.38, 0.94 | 0.19, 0.91 | 0.18, 0.92 | | | | | | | | |
| OST $AUC_g$ | *r* | 0.118 | 0.208 | 0.211 | 0.086 | | | | | | | |
| | 95% CI | -0.49, 0.65 | -0.42, 0.70 | -0.44, 0.72 | -0.51, 0.63 | | | | | | | |
| WEET $AUC_g$ | *r* | 0.532 | 0.599 | .733* | .664* | 0.267 | | | | | | |
| | 95% CI | -0.10, 0.86 | 0.00, 0.88 | 0.24, 0.93 | 0.11, 0.90 | -0.40, 0.75 | | | | | | |
| $Log_{10}$ OGT $Cmax_i$ | *r* | .974** | .879** | .815** | .715** | 0.128 | 0.472 | | | | | |
| | 95% CI | 0.91, 0.99 | 0.62, 0.97 | 0.42, 0.95 | 0.24, 0.91 | -0.48, 0.65 | -0.18, 0.84 | | | | | |
| $Log_{10}$ OST Cmax | *r* | .931** | .986** | .908** | .677* | 0.187 | .604* | .867** | | | | |
| | 95% CI | 0.77, 0.98 | 0.95, 1.00 | 0.68, 0.98 | 0.17, 0.90 | -0.43, 0.69 | 0.01, 0.88 | 0.58, 0.96 | | | | |
| $Log_{10}$ WEET $Cmax_i$ | *r* | .890** | .915** | .987** | .727* | 0.222 | .659* | .866** | .917** | | | |
| | 95% CI | 0.62, 0.97 | 0.70, 0.98 | 0.95, 1.00 | 0.23, 0.92 | -0.44, 0.73 | 0.10, 0.90 | 0.56, 0.96 | 0.70, 0.98 | | | |
| $Log_{10}$ FI | *r* | 0.342 | 0.394 | 0.280 | 0.417 | 0.506 | 0.395 | 0.326 | 0.263 | 0.335 | | |
| | 95% CI | -0.29, 0.77 | -0.23, 0.79 | -0.38, 0.75 | -0.21, 0.80 | -0.10, 0.84 | -0.27, 0.80 | -0.31, 0.76 | -0.37, 0.73 | -0.33, 0.78 | | |
| $Log_{10}$ $CGITins_{45}$ | *r* | .678* | .629* | .659* | .622* | 0.235 | 0.575 | .628* | .580* | .665* | .603* | |
| | 95% CI | 0.17, 0.90 | 0.09, 0.88 | 0.10, 0.90 | 0.07, 0.88 | -0.39, 0.71 | -0.04, 0.87 | 0.09, 0.88 | 0.01, 0.87 | 0.11, 0.90 | 0.04, 0.87 | |
| $CGITPP_g$ | *r* | 0.426 | 0.429 | 0.423 | .741** | 0.195 | .631* | 0.326 | 0.433 | 0.403 | 0.253 | 0.434 |
| | 95% CI | -0.20, 0.80 | -0.19, 0.80 | -0.24, 0.82 | 0.29, 0.92 | -0.43, 0.69 | 0.05, 0.89 | -0.30, 0.76 | -0.19, 0.81 | -0.26, 0.81 | -0.38, 0.72 | -0.19, 0.81 |

a)




b)



c)

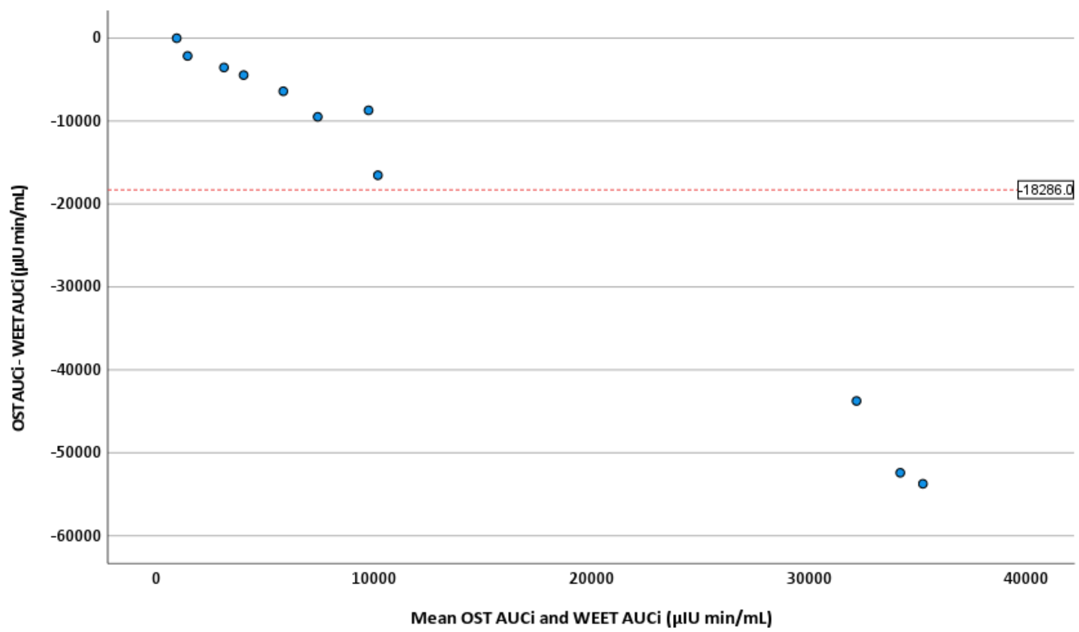



**Fig. 4.2:** Bland Altman plots of agreement for $AUC_i$ between a) OGT-OST, b) OGT-WEET and c) OST-WEET. Dashed red line represents mean bias.

**Table 4.3:** Bland Altman analysis from comparisons of $AUC_i$ for Oral glucose test (OGT) Oral sugar test (OST) and cereal-based test (WEET). LOA, Limit of agreement.

| Comparison | Mean Bias (95% CI) (µIU min/mL) | Coefficient of slope of regression equation (95% CI) | *P* – value for coefficient of slope |
|---|---|---|---|
| OGT $AUC_i$ – OST $AUC_i$ | 33766 (13427 – 54105) | 1.69 (1.6 – 1.8) | <0.0001 |
| OGT $AUC_i$ – WEET $AUC_i$ | 16612 (3562 – 29660) | 0.43 (0.05 – 0.81) | 0.03 |
| OST $AUC_i$ – WEET $AUC_i$ | -18286 (-32356 - -4216) | -1.52 (-1.65 - -1.40 ) | <0.0001 |

### *4.4.2 Insulin and glucose responses*

Insulin responses to the four dynamic tests in individual ponies (1-12) are shown in Fig. 1. There were significant differences in AUCg, AUCi and $Cmax_i$ between the oral tests, with OGT greater than OST and WEET, and WEET greater than OST (Table 1). Based on the CGIT, 4/12 ponies (numbers 1, 7, 10, 12) were classified as IR, 3 with CGIT $ins_{45}$ >82 µIU/mL and 1 with $PP_g$ > 45min.

$AUC_i$ and $Cmax_i$ from all oral tests, $AUC_g$ for OGT and WEET and CGITPPg and CGIT$ins_{45}$ were greater in the IR group. There was a very strong positive correlation (*r* = 0.85 – 0.94) for $AUC_i$ and $Cmax_i$ between OGT, OST and WEET and moderate-strong positive correlation (*r* = 0.58 – 0.68) between CGIT$ins_{45}$ and the $AUC_i$ and $Cmax_i$ of OGT, OST and WEET. There were strong correlations between $AUC_g$ and $AUC_i$ for the OGT (*r* = 0.78) and WEET (*r* = 0.73) (Table 2). Bland Altman plots of $AUC_i$ are shown in Fig. 2. The majority of values were within the 95% limits of agreement. The bias between OGT-OST and OGT-WEET increased as mean $AUC_i$ increased, whereas there was a decreasing bias with increasing $AUC_i$ for OST-WEET. There was a significant slope of the regression equation for all three comparisons (Table 3).

### *4.4.1 Classification of insulin status*

Data for each test used to classify ID status are shown in Table 4. Median $Tmax_i$ for WEET was 120 min, and the optimal diagnostic cut-off for insulin concentration at this time point was 124 µIU/mL, resulting in a sensitivity and a specificity of 100%. Cohen kappa reflected substantial agreement (0.62 – 0.79, *P*<0.05, 2-sided) between binary results from OGT, OST, WEET and CGIT (Table 5). As tests for IR, the sensitivity and specificity of OGT and OST were adequate to good (Table 5). Only one pony (pony 10) was classified as positive for IR using FI, resulting in a sensitivity and specificity of 25% and 100% respectively (Table 6).

**Table 4.4:** Data at time points used for binary IR classification. Boxes shaded pink are classified as positive for insulin dysregulation. OGT, Oral Glucose Test; OST, Oral Sugar Test; WEET, Weetabix cereal test; CGIT, Combined Glucose Insulin Test; FI, Fasting Insulin concentration; $ins_{120}$, insulin concentration at 120 min; PPg, duration (min) of the positive phase of the glucose curve relative to baseline; $ins_{60\ or\ 90}$, the greatest insulin concentration at 60 or 90 min; *, excluded from analysis.

| Pony number | $OGTins_{120}$ (μIU/mL) | $OSTins_{60/90}$ (μIU/mL) | $WEETins_{120}$ (μIU/mL) | FI (μIU/mL) | CGITPPg (min) | $CGITins_{45}$ (μIU/mL) |
|---|---|---|---|---|---|---|
| 1 | 269 | 86 | 161 | 3.7 | 44 | 119 |
| 2 | 163 | 53 | 61 | 2.7 | 26 | 45 |
| 3 | 56 | 22 | 52 | 1.0 | 30 | 31 |
| 4 | 20 | 5 | 15 | 4.1 | 17 | 51 |
| 5 | 78 | 20 | 27 | 0.9 | 20 | 65 |
| 6 | 72 | 5 | 2 | 2.7 | 39 | 28 |
| 7 | 367 | 75 | 164 | 3.8 | 37 | 109 |
| 8 | 53 | 15 | 23 | 0.2 | 29 | 46 |
| 9 | 29 | 19 | 87 | 0.3 | 18 | 24 |
| 10 | 282 | 87 | 195 | 10.4 | 45 | 117 |
| 11 | 111 | 26 | 56 | 1.7 | 22 | 44 |
| 12 | 100 | 25 | 35* | 0.6 | 56 | 51 |

**Table 4.5:** Agreement (Cohen's Kappa coefficient) on binary insulin dysregulation status between oral glucose test (OGT), oral sugar test (OST) and cereal test (WEET), and combined glucose insulin test (CGIT).

| Comparison | Cohen's Kappa | 95% CI | *P* - value |
|---|---|---|---|
| OGT-OST | 0.67 | 0.27 - 1 | 0.01 |
| OGT-WEET | 0.62 | 0.18 - 1 | 0.03 |
| OGT-CGIT | 0.67 | 0.27 - 1 | 0.01 |
| OGT-FI | 0.17 | -0.14 – 0.48 | 0.30 |
| OST-WEET | 0.79 | 0.41 - 1 | 0.01 |
| OST-CGIT | 0.63 | 0.15 - 1 | 0.03 |
| OST-FI | 0.31 | -0.18 – 0.8 | 0.14 |
| WEET-FI | 0.42 | -0.17 - 1 | 0.09 |
| CGIT-FI | 0.31 | -0.18 – 0.8 | 0.14 |

**Table 4.6:** Sensitivity and specificity of Oral glucose test (OGT), Oral sugar test (OST, Fasting insulin (FI) for a diagnosis of insulin resistance, using results from the combined glucose insulin test (CGIT) as the reference / gold standard test. The cereal test (WEET) is not included as the diagnostic cut-off was established against the same CIGIT results.

| Test | Sensitivity (%) | Specificity (%) |
|---|---|---|
| OGT | 100 | 75 |
| OST | 75 | 88 |
| FI | 25 | 100 |

## 4.5 Discussion

To our knowledge, this study is the first to directly compare four established diagnostic tests for ID; the FI, OGT, OST and CGIT as well as a cereal-based test in ponies. The results show that the oral carbohydrate tests, irrespective of the source of carbohydrate (glucose, corn syrup or wheat cereal), were very strongly correlated when using $AUC_i$ or $Cmax_i$ and showed reasonable agreement across a range of insulin dysregulation. Proportional bias for $AUC_i$ was present, particularly for comparisons with OST which is likely due to the short duration insulin peak with this test compared to the more sustained insulinaemic responses with OGT and WEET, particularly for ponies with ID. When cut-offs were applied, the kappa coefficients were lower reflecting that the single or dual points used in deriving cut-offs reduce the diagnostic value of the tests. This may, in part, be due to the different rates of consumption in different ponies and different techniques employed to derive the cut-offs used in this study.

The $AUC_i$ and $Cmax_i$ of the oral carbohydrate tests were only moderately to strongly correlated with the $CGITins_{45}$ and $CGITPP_g$ of the CGIT despite substantial kappa agreement between OGT and CGIT and OST and CGIT when using the cut-offs. This is likely to reflect the role of gastrointestinal incretins in the response to the oral tests which would be absent in the intravenous CGIT (de Laat et al., 2016), also the dichotomous result for the CGIT was taken as a positive from either $CGITins_{45}$ or $CGITPP_g$. When using the oral tests three ponies would have tested positive using the cut-offs for the WEET (and CGIT which was used to define the cut-off for WEET), four for the OST and six for the OGT. Research investigating the incretin response to all these oral tests for ID is warranted and might aid in clinical interpretation.

The fact that the oral tests were so strongly correlated to each other, and that the insulinaemic response and kappa value are less with the CGIT due to a lack of gastrointestinal incretin stimulation, supports that the reference standard should be an oral test. It is likely that the OGT, with the greatest sensitivity for detection, is the better reflection of ID in these ponies. If the cut-off for WEET had been determined using the OGT as a reference standard 6 horses out of 11 (since horse 12 had to be excluded for not eating the WEET) would have tested positive for ID, meaning this test is potentially the most sensitive test. In Fig. 1 the variation in insulinaemic responses was far greater in the oral tests, especially the two that delivered 1g/kg CHO (WEET and OGT), further emphasising the likely role of incretins in the

response to oral CHO. Variability in time to consume the feed in the OGT and WEET could account for this too.

Unfortunately, the palatability of WEET was poor with half the horses only consuming two thirds of the ration and one horse only consuming one third and having to be excluded. A smaller meal of WEET would likely have resulted in a greater percentage consumption and reduced the average time to consume the meal, resulting in less variability in the peak insulin concentration. A study by de Laat and Sillence (de Laat and Sillence, 2017) included a comparison of 1g/kg and 0.75g/kg OGTs in a group of eight ponies. Unexpectedly, the average insulin response was greater following the 0.75g/kg dose compared to the 1g/kg dose, suggesting that a smaller meal might result in faster consumption, and that maximal insulin secretion occurs at doses of oral CHO lower than 1g/kg. Further research on optimal oral CHO dose and more palatable options for cereal tests is warranted, especially as this form of oral dynamic test may potentially have greater sensitivity for detection of ID despite the palatability issues.

The lower insulinaemic response to the OST could be explained simply by the lower dose of CHO, but it is interesting to note that the $AUC_g$ to the OST was only weakly correlated to the $AUC_i$, ($r = 0.208$) which is in contrast to the OGT in which the correlation was strong ($r = 0.783$). It may be that corn syrup is less glycaemic and this may affect its diagnostic ability. Additionally, there is no prehension or mastication of feed during the OST, which might reduce enteric reflexes stimulating gastrointestinal motility, secretion and absorption. The OST was evaluated against the frequently sampled glucose and insulin tolerance test (FSGITT) and CGIT in a previous study (Dunbar et al., 2016), and similarly, the glucose response to the OST failed to differentiate insulin resistant and sensitive horses, in contrast to the CGIT and FSGITT. The low sensitivity of the 0.15ml/kg OST used in this study is supportive of other studies (Jocelyn et al., 2018) which have proposed increasing the dose of corn syrup to increase the sensitivity of the test.

Previous studies have relied on cut-offs to attempt to define the sensitivity and specificity of different diagnostic tests, but as we have shown in this study with WEET, results derived from a test that involves both gastrointestinal incretin response as well as a response to intravenous glucose appear to be more sensitive for diagnosis of ID relevant to the situation in the field. Since ID is the central feature of EMS and endocrinopathic laminitis in horses, the most sensitive test is warranted.

Dynamic intravenous tests such as the FSIGTT and euglycaemic hyperinsulinaemic clamp have been referred to as 'gold standard' tests for IR (Dunbar et al., 2016, Lindåse et al., 2021, Pratt-Phillips et al., 2015), but there remains no equivalent status test for ID which includes the contribution of the enteroinsular axis. Longitudinal studies have evaluated different tests for prediction of naturally occurring (Menzies-Gow et al., 2017, Treiber et al., 2006) and experimental laminitis (Meier et al., 2018), and have established cut-offs for various tests based on these outcomes. These have provided useful data for predicting laminitis risk, but some of the tests or assays used are now unavailable (Menzies-Gow et al., 2018) and there has been conflicting data on the predictive values of some of these tests (Lindåse et al., 2021). The lack of an accepted 'gold standard' test for ID means that in this study the cut-offs used might not be optimal for the diagnosis of EMS and laminitis risk.

Fasting was originally recommended to standardise basal insulin values (Frank et al., 2010), but more recent research (Lindåse et al., 2021, Olley et al., 2019) has shown that fasting in horses results in very low insulin values, even in horses with ID, so cut-offs previously employed (Dunbar et al., 2016, Frank and Geor, 2014, Frank et al., 2010) are no longer valid. However, even employing a lower cut-off of 5.4 µIU/mL based on the work by Olley (Olley et al., 2019), the sensitivity of FI in this study was poor as a single diagnostic test, with only one additional horse being diagnosed as ID-positive than if the conventional cut-off (20 µIU/mL) had been used. Duration of fasting has been associated with IR (Bertin et al., 2016), meaning that longer periods of fasting could reduce the specificity of tests. The utility of basal insulin in the naturally fed state is likely to be greater (Durham et al., 2019) and research on insulinaemic responses following forage feeding has shown it is able to differentiate between ID and normal horses (Chapter 5). Marked difference in insulinaemic response to forages with different NSC content (Borgia et al., 2011, Lindase et al., 2018) can limit the accuracy of this approach, however, and further work based on this is warranted so that ID status in horses can be diagnosed and monitored with their usual diets.

Non-linear relationships have been demonstrated between measures of insulin resistance and the insulinaemic response to oral carbohydrates in other studies (Lindase 2017), and it is possible that with a larger data set, the current study might have shown a similar association. Correlation between a large number of different variables was examined in this study (Table 2), without adjustment of the significance level for multiple testing. This was done to balance the risk of type 1 and type 2 errors, and results should be interpreted accordingly.

In conclusion, the three oral dynamic tests of ID using glucose, corn syrup and wheat cereal, were highly correlated with each other when using $AUC_i$ and $Cmax_i$, showed adequate agreement of $AUC_i$ and good agreement of dichotomised results. They were more sensitive than the CGIT in detecting ponies with ID presumably due to their ability to elicit an incretin response as well as a response to the absorbed glucose. The wheat cereal-based test showed a high sensitivity for diagnosis of ID, but was poorly palatable while fasted basal insulin was the least sensitive test. More work to ensure diagnostic cut-offs are derived from appropriate methods is warranted.

## 4.6 Manufacturers' Addresses

[1] Leominster, UK

[2] Milton Keynes, UK

[3] Burton Latimer, UK

[4] Cordova, Tennessee, USA

[5] Basingstoke, Hants, UK

[6] Camberley, Surrey, UK

[7] Dorset, UK

[8] Yellow Springs, OH, USA

[9] New York, USA

[10] Ostend, Belgium

# Chapter 5: Insulinaemic and glycaemic responses to three forages in ponies

A modified version of this chapter has been published as a paper

(See Supplementary information)

Carslake, H. B., Argo, C. McG., Pinchbeck, G.L., Dugdale, A. H. A., McGowan, C. M. (2018)

Insulinaemic and glycaemic responses to three forages in ponies

*The Veterinary journal* 235, 83-89

# 5.1 Abstract

Reduction of the hyperinsulinaemic response to feeding is central to the management of insulin dysregulation (ID). The aim of this study was to compare insulinaemic and glycaemic responses to soaked hay, hay and haylage. Twelve ponies of mixed breeds were recruited and maintained under identical management conditions. A randomised 4-way crossover trial was conducted in which fasted animals were either meal-fed 0.25% body weight as dry matter intake soaked hay, hay or haylage, or administered an oral glucose test (OGT). Blood glucose and serum insulin concentrations were measured before, and at 2 h following OGT and regularly for 5 h following forage meals.

Median [IQR] area under the curve (AUC) for insulin ($AUC_i$) was greater for haylage (6495 [17352]) vs hay (2932 [5937]; $P = 0.019$) and vs soaked hay (1066 [1753]; $P = 0.002$), and greater for hay vs soaked hay ($P = 0.002$). AUC for glucose ($AUC_g$) was lower for soaked hay (1021 [99]) vs hay (1075 [105]; $P = 0.002$) and vs haylage (1107 [221]; $P = 0.003$). Six ponies were classified as having ID based on OGT result. $AUC_i$ was greater in ID vs non-ID ponies after all forages. In contrast, there was no detectable effect of ID status on $AUC_g$. On an equivalent DM basis, soaked hay produced the lowest and haylage the highest insulinaemic and glycaemic responses to feeding. The insulinaemic effects of all forages were greater in ponies with ID. These data support the soaking of hay to reduce postprandial insulinaemic responses in ponies.

## 5.2 Introduction

Insulin dysregulation (ID) is a term that refers collectively to insulin resistance and hyperinsulinaemia, both of which can lead to exaggerated hyperinsulinaemic responses to dietary carbohydrates (Frank and Tadros, 2014). Hyperinsulinaemia has been shown to induce laminitis in experimental studies (Asplin et al., 2007), and is associated with the development of laminitis in field studies (Menzies-Gow et al., 2017, Treiber et al., 2006, Walsh et al., 2009a). Risk factors for ID include pony breed and obesity (Frank and Tadros, 2014).

Dietary modification to reduce hyperinsulinaemia, particularly after feeding, is central to the management of ID. Consistent improvements in insulin sensitivity were achieved in several studies through dietary restriction using hay- or soaked hay-based feedstuffs (Argo et al., 2015, Dugdale et al., 2010, McGowan et al., 2013a, Morgan et al., 2016a). Water soaking of hay substantially decreases water soluble carbohydrate (WSC) content (Argo et al., 2015, Longland et al., 2011, Mack et al., 2014) and could be expected to elicit a correspondingly decreased glycaemic and insulinaemic response on feeding. However, soaking hay is not always practical, and results in loss of water-soluble macro-minerals (Argo et al., 2015). Haylage, which is fermented conserved grass with characteristically high moisture content, may represent an alternative low-glycaemic diet for use in weight loss protocols and longer term management of ID. WSC content of forages can vary greatly according to grass type, and conditions of harvesting and storage (Longland et al., 2011, Müller, 2009), however data suggest that the WSC content of haylage is commonly lower than that of dry hay (Müller and Udén, 2007) and may conserve concentrations of other nutrients lost through soaking.

Forages causing lower glycaemic and insulinaemic responses in weight loss protocols can aid the resolution of ID (McGowan et al., 2014). In animals where weight loss has been achieved, long-term feeding of forages with reduced glycaemic and insulinaemic responses may maintain a reduced laminitis risk. It is important that an evidence-base is established to critically evaluate the glycaemic and insulinaemic responses of ponies to commonly available forages and across the expected range of insulin dysregulation. In this study we hypothesised that the glycaemic and insulinaemic response to haylage would be greater than that of soaked hay, but less than that of dry hay. Therefore, the aim of this study was to compare insulinaemic and glycaemic responses of ponies to three commonly fed forages: soaked hay, hay and haylage.

# 5.3 Materials and methods

### *5.3.1 Animals*

Twelve ponies that had previously been maintained on a combination of preserved forages and pasture were purchased locally. To ensure that animals with a wide range of ID were included, ponies were screened between 9 and 44 days before the start of the study using either a combined glucose-insulin tolerance test (CGIT) (Eiler et al., 2005) (n = 10) or basal insulin (n = 2). Six ponies were selected with ID (basal serum insulin concentration >60µIU/mL or serum insulin concentration >100 µIU/mL at 45 min or >20 µIU/mL at 75 min post-CGIT) and six ponies without ID were selected.

Animals were excluded if they showed signs of pituitary par intermedia dysfunction (PPID; hypertrichosis, muscle atrophy), or clinical laminitis (grade >0) (Obel, 1948). Animals were confirmed to be free of other confounding disease by clinical examination, haematology, oral examination and, in animals ≥10 years of age, a basal adrenocorticotrophic hormone (ACTH) concentration < 29pg/mL. All ponies were dewormed on recruitment. The study was conducted under the Animals (Scientific Procedures) Act 1986 (project licence PPL 40/3715, date of approval 6th September 2013).

Using the sample size of 12 ponies calculated for the study in Chapter 4, a prospective power calculation was performed. This showed there was 80% power to detect a 25% difference in the $AUC_g$ from an initial value of 1105 mmol/L/min and a standard deviation of 170 mmol/L/min, with values anticipated from clinical experience. Use of a crossover design increased the power to 95%.

### *5.3.2 Housing and feed*

Ponies were habituated to handling, stabling, feeding and weighing for 2 weeks prior to the start of the study. During this period and throughout the study the ponies were individually stabled on wood shavings for 22 h/day and fitted with a closed grazing muzzle (Shires Equestrian[1]) and turned out into a 0.3 ha paddock for free exercise for 2 h/day. Daily feed intake was standardised at 2% body weight (BW) fresh weight, same batch, dry meadow hay provided as two daily meals (0.5% BW at 12:00 after turn-out and 1.5% BW at 17:00). A proprietary feed balancer (Lite Balancer, Spillers[2]) was provided (200g/pony/day).

### 5.3.3 Forages

Hay and soaked hay from a single large bale and haylage (West Lancs Haylage[3]) from a single batch and pallet were used for the study. All forages consisted of mixed grass species and were from the same region (Northwest England). All forage feeds used in the study were prepared by mixing multiple random grab samples. Haylage was preserved as small (14kg) individually wrapped bales and a new bale was opened for each test feed date. Soaked hay was produced by mixing random grab samples of dry hay from the single large bale in a hay net and soaking by complete immersion in water at ambient temperature for 14 h (Mack et al., 2014), followed by hanging to drain for 30 min. At the start of the study, for each forage, multiple grab samples were mixed and a sample (500g) analysed for dry matter (DM) in triplicate by oven-drying (70°C) until constant mass. Wet chemistry analyses of acid detergent fibre (ADF), neutral detergent fibre (NDF), crude protein (CP), ash, starch, WSC and gross energy (GE) were performed in duplicate on dried, ground forage homogenates by an approved laboratory (Sciantec Analytical Services[4]). Non-structural carbohydrate was calculated as WSC + starch. Digestible energy (DE) was estimated according to a calculation described by Pagan (1998) (Pagan, 1998), assuming 1.2% fat.

### 5.3.4 Study protocol

The data in this chapter were extracted from a larger, 7-way cross over study, as described in Chapter 4. Every week during the habituation period and throughout the study, ponies were weighed (Horse Weigh), body condition score (BCS) was estimated on a scale of 1 - 9 (Kohnke, 1992), a cresty neck score (CNS) was assessed (Carter et al., 2009a), and girth and belly (widest point of the abdomen) circumference (cm) were measured. At intervals of at least 7 days during the study, all 12 ponies received a general clinical examination, and a catheter was placed in the jugular vein. That evening, hay provision was restricted (1% BW fed at 5pm) to ensure complete consumption by midnight. The following morning, between 6am and 7am, a baseline blood sample (t=0) was obtained and then a meal of either 0.25% BW DM soaked hay (weighed after soaking/draining), hay, or haylage, or an oral glucose test (OGT) (Borer et al., 2012, Frank and Geor, 2014, Smith et al., 2016) was administered. The interventions used in this chapter constituted a four-period, four treatment, cross-over design with the order of the forage meals and OGT for each pony determined by simple randomisation using randomisation software.

The OGT consisted of 1 g/kg BW glucose mixed with 1g/kg BW chaff-based feed (Happy Hoof, Spillers[2]) and 1 mL/kg BW water. Blood samples were obtained from the jugular catheter 30, 60, 90, 120, 150, 180, 240 and 300 min after administration of the forage, and 120 min after the OGT. Time from T0 until complete consumption of feeds was recorded. After the last blood sample the catheter was removed, and there was a minimum 6-day wash out period until the next forage test or OGT.

### *5.3.5 Serum insulin and blood glucose analysis*

Glucose and insulin were measured for all blood samples. Blood for insulin analysis was collected into plain tubes and left at room temperature to clot for 1-2 h, after which it was centrifuged at 2000 *g* for 10 min and the serum separated and stored at 5°C until analysis. Insulin concentrations for all serum samples were measured in duplicate within 8 h of collection by a chemiluminescent immunoassay (Immulite 2000, Siemens[5]) validated for use in horses with in intraassay coefficient of variation (CV) between 1.8 – 2.4% and an interassay CV between 3.0 – 7.1% (Chapter 3). Any samples with an insulin concentration greater than the upper range of the assay (>300 µIU/mL) were diluted 1:1 with activated charcoal (Sigma-Aldrich Ltd[6]), stripped equine serum (Chapter 3) and reanalysed. Blood for the evaluation of glucose concentrations was collected into fluoride oxalate tubes and kept on ice until analysis within 8 h. Whole blood glucose was measured using a glucose oxidase enzymatic method (YSI 2300 STAT[7]).

### *5.3.6 Data analysis*

Statistical analysis was performed using SPSS version 22 (IBM[8]). Insulin dysregulation was defined as a serum insulin concentration >85 µIU/mL at t = 120 min during the OGT (Frank and Geor, 2014, Smith et al., 2016). Area under the curve for insulin ($AUC_i$) and glucose ($AUC_g$) were calculated using the trapezoid method with 0 µIU/mL as the baseline. Maximum concentrations of insulin ($Cmax_i$) and glucose ($Cmax_g$) were derived from concentration over time curves.

Continuous data were tested for normality using the Shapiro-Wilk test and reported as mean ± standard deviation (SD) or median and interquartile range (IQR) depending on distribution. Differences in repeated measures between the three forages were analysed using a repeated measures analysis of variance (ANOVA) for normally distributed data (body measurements and time to consume) or the Friedman test for non-parametric data (glycaemic and insulinaemic responses), followed by a post-hoc paired *t* test or Wilcoxon signed-rank test,

respectively. Comparisons between ID and non-ID animals were performed using the Mann Whitney *U* test. Significance (2-sided) was set at $P < 0.05$.

### 5.3.7 Dates and location of the study and the candidate's role

The cross over study, including analysis of samples took place in 2014 at the University of Liverpool. The candidate contributed to study design and organised and performed data collection and sample analysis with assistance from technicians. He performed data analysis and prepared this chapter of the thesis.

## 5.4 Results

### 5.4.1 Animals

All ponies were mixed, native British breeds, comprising 11 mares and 1 gelding, aged 9.1 ± 3.4 years with a body weight of 280 ± 49 kg. Median (IQR) BCS was 7.2 (6.0 – 7.7) out of 9, with a range of 4.2-8.5 (Appendix 1). On the basis of the OGT, six ponies were classified as ID and six as non-ID (Appendix 2). There was no significant difference in mean body weight, girth or belly circumference, CNS or BCS at the time each forage type was fed, or between ID and non-ID pony groups (Appendix 1). Over the study period, mean body weight increased from 278 ± 51.2 kg to 284 ± 52.6 kg ($P = 0.001$).

### 5.4.2 Forage analysis and consumption of feeds

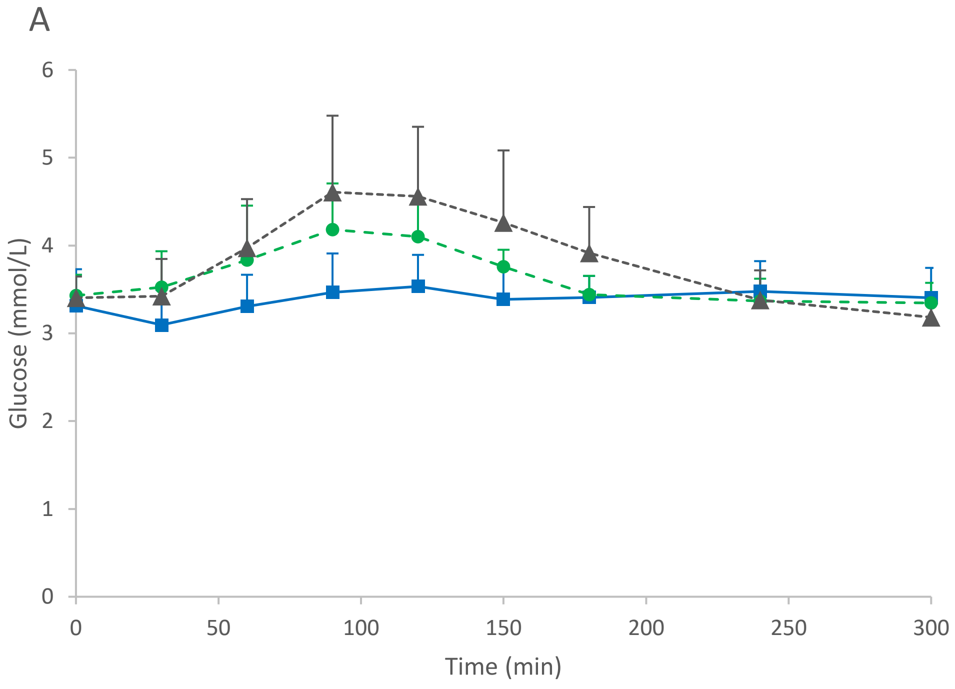
The results of wet chemistry feed analysis for each forage on a DM basis are shown in Table 1. All ponies consumed the entire test feed offered for all forages (dry hay, soaked hay and haylage) on each occasion. Three ponies did not finish the glucose/chaff mix during the OGT, consuming 85%, 93% and 94% of the available feed, which was considered insignificant to the result of the test. There was no difference in time to consume the different forages or effect of ID status on time to consume any of the feeds. (Appendix 3).

**Table 5.1** Wet chemistry analyses on a dry matter basis, of forages from duplicate samples, (mean ± SD)

| Feed | Soaked hay | Dry Hay | Haylage |
|---|---|---|---|
| DM (%) | 24.7 ± 1.8 | 87.5 ± 0.02 | 58.4 ± 1 |
| Acid detergent fibre (%) | 41.1 ± 0.1 | 39.1 ± 2.4 | 29.6 ± 1.1 |
| Neutral detergent fibre (%) | 72.1 ± 3.5 | 65.9 ± 0.9 | 52 ± 1.6 |
| Crude protein (%) | 6.0 ± 0.2 | 6.2 ± 0.4 | 8.0 ± 0.1 |
| Ash (%) | 3.0 ± 0.4 | 3.6 ± 1.2 | 5.8 ± 0.1 |
| Starch (%) | 0.4 ± 0.5 | 2.3 ± 3.2 | 2 ± 2.8 |
| Water soluble carbohydrate (%) | 10.6 ± 2 | 13.8 ± 0.6 | 16.5 ± 6.9 |
| Non-structural carbohydrate (%) | 10.9 ± 2.5 | 16.0 ± 2.6 | 18.5 ± 4.1 |
| Gross energy (MJ/Kg) | 17.3 ± 0.2 | 17.6 ± 1.1 | 17.5 ± 0 |
| Digestible energy (calculated) (MJ/Kg) | 7.9 ± 0.2 | 8.4 ± 0.2 | 8.9 ± 0.3 |

### *5.4.3 Glycaemic and insulinaemic responses to different forages in all ponies*

There was no difference in mean basal (t = 0 min) glucose and insulin concentrations between the times the different forages were fed (Fig. 1). $AUC_g$ was less after feeding soaked hay than after feeding dry hay ($P$ = 0.005) or haylage ($P$ = 0.003; Table 2; Fig. 1A). $Cmax_g$ after feeding haylage was greater than after feeding dry hay ($P$ = 0.037) or soaked hay ($P$ = 0.002), and greater after feeding dry hay than after feeding soaked hay ($P$ = 0.003) (Table 2; Fig. 1A). Feeding haylage resulted in a greater $AUC_i$ and $Cmax_i$ than feeding dry hay ($P$ = 0.02 and 0.03, respectively) or soaked hay ($P$ = 0.002 for both). Feeding dry hay resulted in a greater $AUC_i$ and $Cmax_i$ than feeding soaked hay ($P$ = 0.002 for both), (Table 2; Fig. 2).



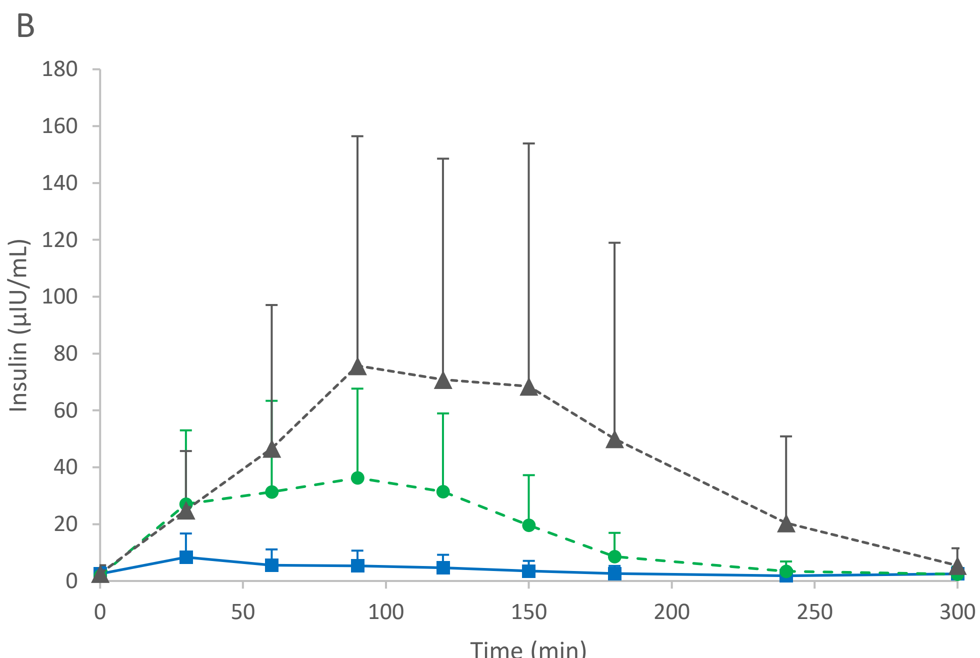




**Fig. 5.1:** Mean ± SD blood glucose (A) and serum insulin (B) concentrations in all ponies (n = 12) after feeding 0.25% body weight dry matter soaked hay (blue squares), dry hay (green circles) and haylage (grey triangles).

**Table 5.2**: Median (IQR) area under the curves for glucose ($AUC_g$) and insulin ($AUC_i$) concentrations over time, and maximum concentration of glucose ($Cmax_g$) and insulin ($Cmax_i$) for all (n = 12), insulin dysregulated (ID, n = 6) and non - ID (n = 6) ponies, after feeding 0.25% body weight dry matter soaked hay, dry hay and haylage.

| Forage | Parameter | All (n = 12) | ID (n = 6) | Non - ID (n = 6) |
|---|---|---|---|---|
| Soaked hay | $AUC_g$ (mmol·min /l) | 1021 (969 - 1069)$^{ab}$ | 1006 (938 - 1090)$^{fg}$ | 1030 (973 - 1070)$^{n}$ |
| | $Cmax_g$ (mmol/l) | 3.7 (3.5 – 3.8)$^{c}$ | 3.7 (3.5 – 3.8)$^{hi}$ | 3.7 (3.5 – 3.8)$^{op}$ |
| | $AUC_i$(µIU·min /mL) | 1066 (236 - 1989)$^{d}$ | 1922 (1440 - 2742)$^{jk}$* | 259 (113 - 436)$^{qr}$* |
| | $Cmax_i$ (µIU/mL) | 4.7 (3.6 – 17.7)$^{e}$ | 15.5 (8.5 – 21.7)$^{lm}$* | 3.7 (2.6 – 3.9)$^{st}$* |
| Dry Hay | $AUC_g$(mmol·min /l) | 1075 (1033 - 1139)$^{a}$ | 1061 (1001 - 1135)$^{f}$ | 1078 (1037 - 1149) |
| | $Cmax_g$(mmol/l) | 4.1 (4 – 4.3)$^{c}$ | 4.2 (3.9 – 4.5)$^{h}$ | 4.1 (4.0 – 4.5)$^{o}$ |
| | $AUC_i$(µIU·min /mL) | 2932 (1739 - 7677)$^{d}$ | 7645 (4582 - 12665)$^{j}$* | 1773 (974 - 1950)$^{q}$* |
| | $Cmax_i$ (µIU/mL) | 26 (12.7 – 59.1)$^{e}$ | 57.6 (38.3 – 89.8)$^{l}$* | 13 (11.6 – 19.1)$^{s}$* |
| Haylage | $AUC_g$(mmol·min /l) | 1107 (1055 - 1276)$^{b}$ | 1107 (1055 - 1298)$^{g}$ | 1117 (1031 - 1255)$^{n}$ |
| | $Cmax_g$ (mmol/l) | 4.5 (4.1 – 5.4)$^{c}$ | 4.7 (4.1 – 5.6)$^{i}$ | 4.5 (4.2 - 5.1)$^{p}$ |
| | $AUC_i$ (µIU·min /mL) | 6495 (2379 - 19732)$^{d}$ | 18569 (6136 - 30993)$^{k}$* | 3267 (1511 - 7181)$^{r}$* |
| | $Cmax_i$ (µIU/mL) | 51 (17 - 143)$^{e}$ | 127.8 (43.6 – 230.6)$^{m}$* | 26 (11.3 – 57.7)$^{t}$* |

$^{a-s}$ Corresponding values in the same column with the same superscript letters are significantly different (*P*<0.05, 2-sided). * Values are significantly different (*P*<0.05, 2-sided) between ID and non-ID ponies.

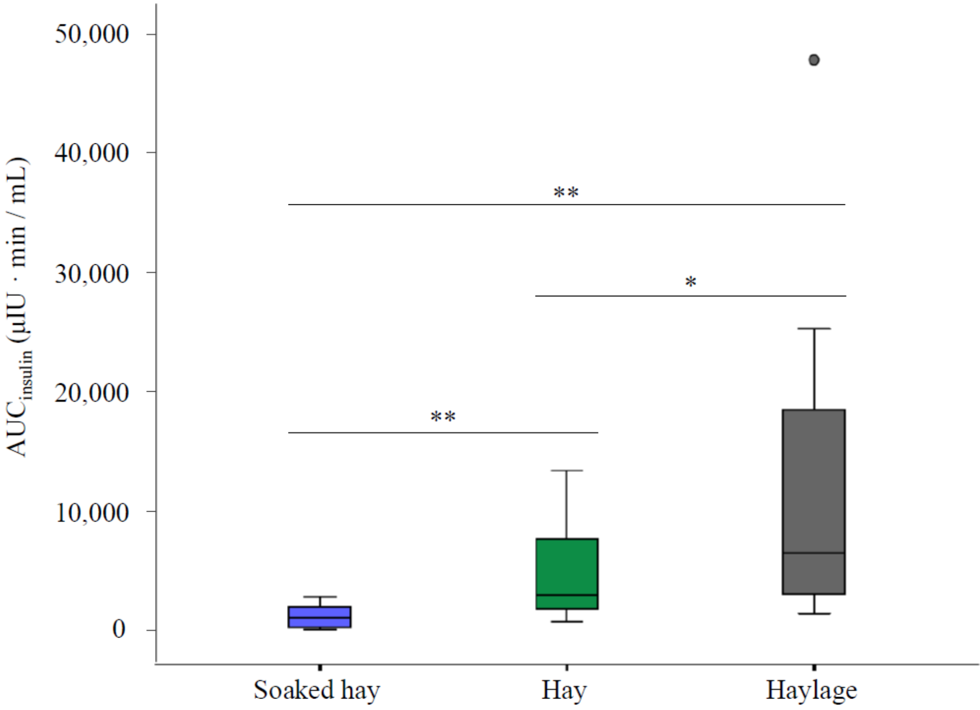


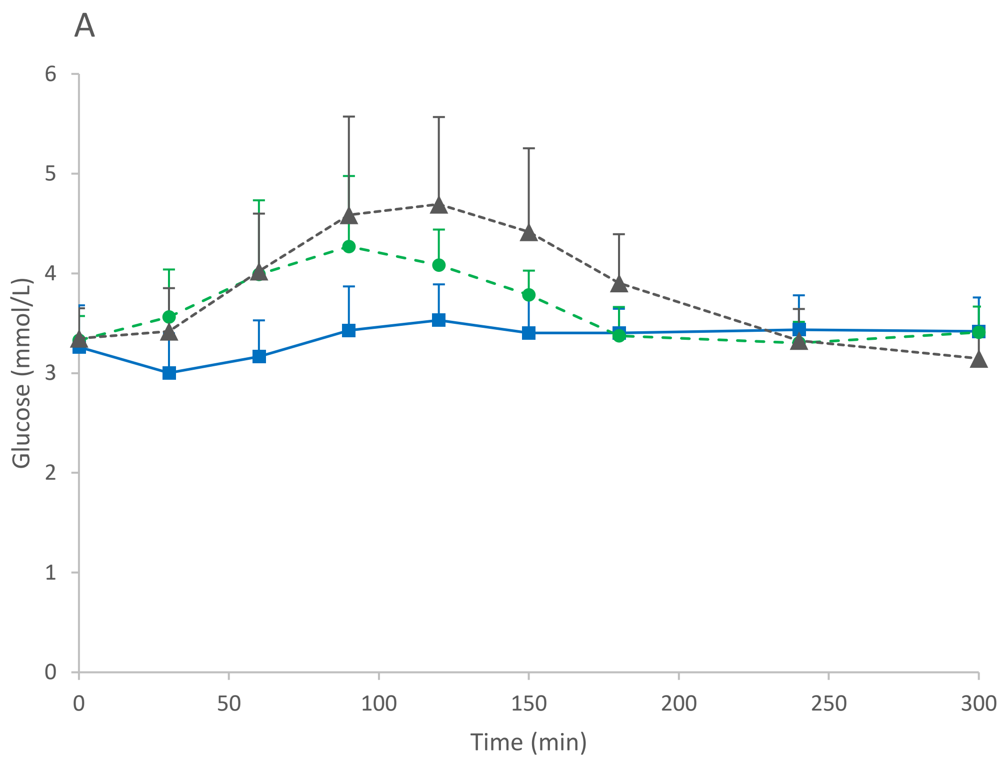

**Fig. 5.2:** Box-and-whisker plot showing area under the curve for the insulin/time ($AUC_i$) curve after feeding 0.25% body weight dry matter soaked hay (blue), dry hay (green) and haylage (grey) in all ponies (n=12). Data for all three forages differed significantly. (* = $P$<0.05, ** $P$<0.01). The bar denotes median and the box is interquartile range.

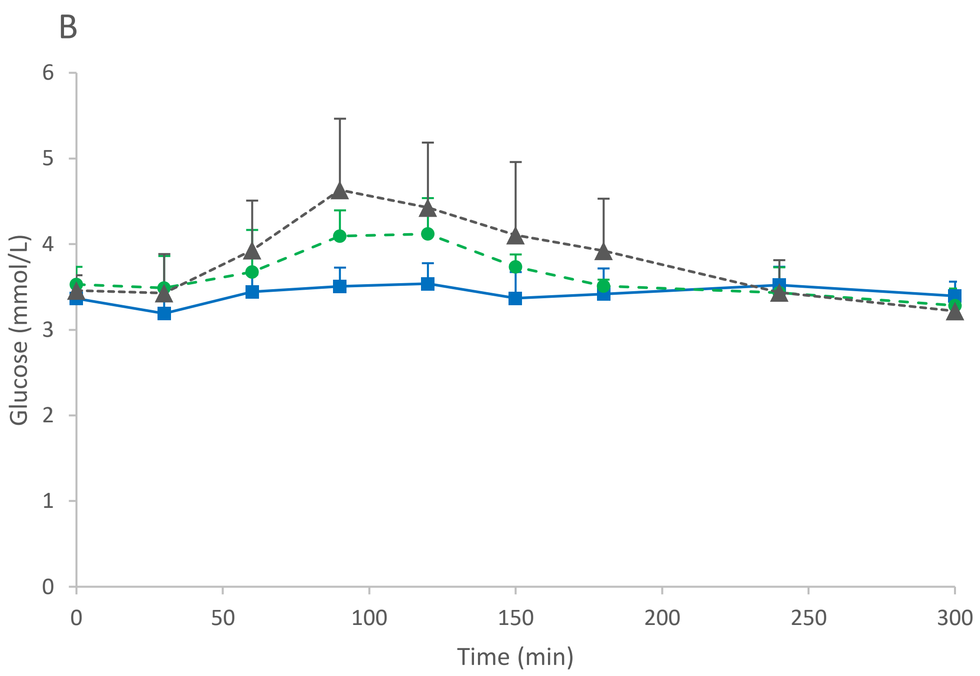
### *5.4.4 Glycaemic and insulinaemic responses within insulin dysregulated and non-insulin dysregulated groups*

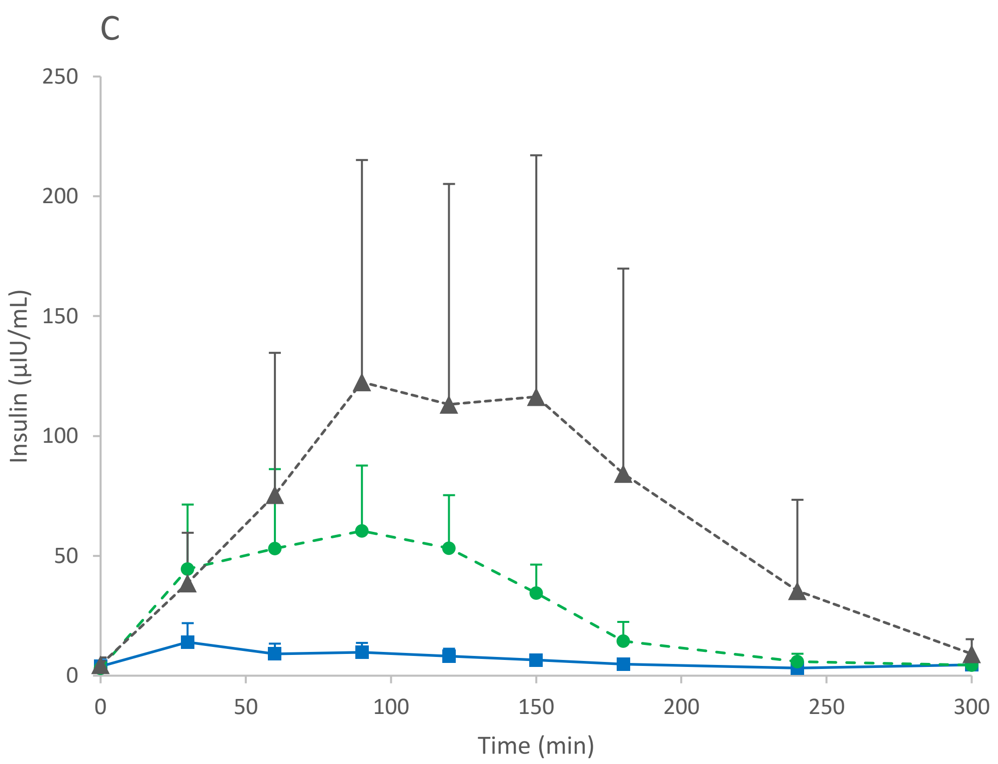
Glycaemic and insulinaemic responses to the three different forages in ID and non - ID ponies are shown in Fig. 3. In ID ponies, both $AUC_g$ and $AUC_i$ were less after feeding soaked hay than after feeding hay ($P$ = 0.028 for both) or haylage ($P$ = 0.046 and $P$ = 0.028, respectively; Table 2). Feeding soaked hay resulted in lower $Cmax_g$ and $Cmax_i$ than feeding dry hay ($P$ = 0.028 for both) and haylage ($P$ = 0.028 for both, Table 2). In the non - ID group, $AUC_g$ was greater after feeding haylage than after feeding soaked hay ($P$ = 0.028). $AUC_i$ was less after feeding soaked hay than after feeding dry hay or haylage ($P$ = 0.028 for both). $Cmax_g$ and $Cmax_i$ were both less after feeding soaked hay than after feeding dry hay ($P$ = 0.06 and $P$ = 0.028, respectively) or haylage ($P$ = 0.028 for both, Table 2; Figs. 3 and 4).

### *5.4.5 Glycaemic and insulinaemic responses between insulin dysregulation groups*

Basal insulin concentration in all tests was greater in ID ponies (median 3.42 µIU/mL; IQR 3.51 µIU/mL) than non - ID ponies (median 0 µIU/mL; IQR 1.05 µIU/mL; $P < 0.001$). There was no difference in mean ± SD basal glucose concentration between ID (3.31 ± 0.32 mmol/L) and non - ID (3.45 ± 0.19 mmol/L) ponies ($P \geq 0.05$). Ponies with ID had a greater $AUC_i$ than non - ID ponies for soaked hay ($P$ = 0.002), dry hay ($P$ = 0.002) and haylage ($P$ = 0.026, Table 2; Fig. 4). $Cmax_i$ was greater in ID ponies than non - ID ponies for soaked hay ($P$ = 0.002), hay ($P$ = 0.002) and haylage ($P$ = 0.041). There was no detectable effect of ID status on $AUC_g$ or $Cmax_g$ for any of the forages.


A
Glucose (mmol/L)
6
5
4
3
2
1
0
0
50
100
150
200
250
300
Time (min)


B
Glucose (mmol/L)
6
5
4
3
2
1
0
0
50
100
150
200
250
300
Time (min)



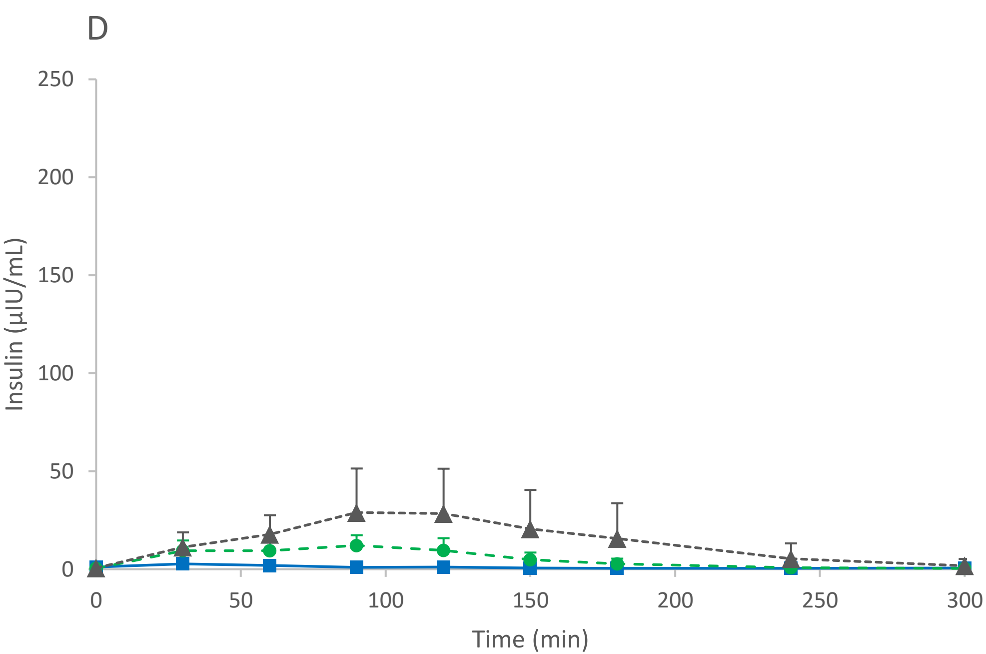




**Fig. 5.3:** Mean ± s.d. blood glucose (A and B) and serum insulin (C and D) concentrations in insulin dysregulated (ID, A and C; n = 6) and non - ID (B and D; n = 6) ponies after feeding 0.25% BW DM soaked hay (blue, squares), hay (green circles) and haylage (grey triangles).

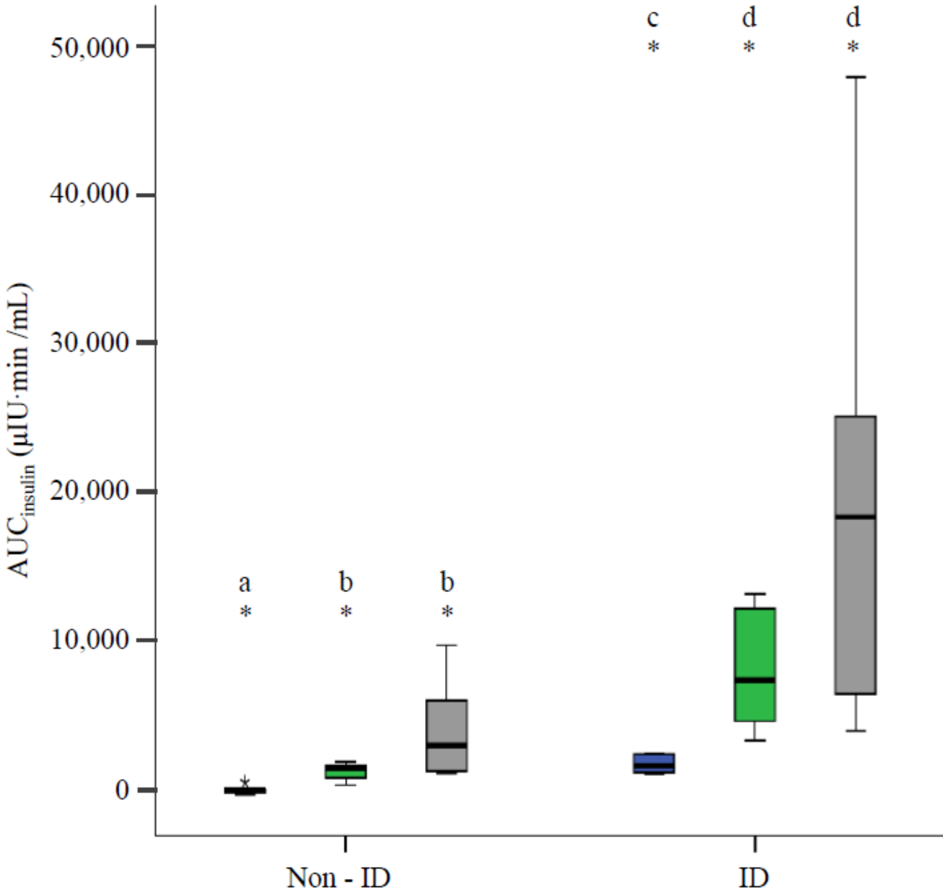



**Fig. 5.4**: Box-and-whisker plot showing area under the curve for insulin/time curve ($AUC_i$) after feeding 0.25% BW DM soaked hay (blue), hay (green) and haylage (grey), in insulin dysregulated (ID, n = 6) and non - ID (n = 6) ponies. The bar denotes median and the box is interquartile range. Different letters indicate significant difference ($P$<0.05, 2-sided) between forages. * Indicates significant difference ($P$<0.05, 2-sided) between ID and non-ID groups.

## 5.5 Discussion

In this study, it was demonstrated that soaked hay, dry hay and haylage can result in significantly different insulinaemic and glycaemic responses when fed on an equivalent DM basis to ponies with a wide range of insulin sensitivity. The longer-term benefit of dietary restriction with low NSC feeds on insulin sensitivity has been demonstrated previously (McGowan et al., 2013a, Morgan et al., 2016a), but this is the first study to compare the immediate postprandial effects of these different types of commonly fed forages.

The lower glycaemic and insulinaemic responses after consuming soaked hay compared to dry hay and haylage in this study were supportive of our original hypothesis. Soaking hay in water for a minimum of 3 h causes a variable but significant reduction in NSC content (Longland et al., 2014, Mack et al., 2014). In the present study, it is likely that the reduced NSC content of the soaked hay was the main cause of the reduced glycaemic, and therefore insulinaemic, response, but other factors may have contributed. Although the average rate of DM consumption in the current study was not significantly lower for soaked hay than for other forages, in previous studies the rate of forage consumption was higher for ensiled forage (Müller and Udén, 2007), and forages with a higher NSC content (Borgia et al., 2011). More rapid consumption could potentially result in earlier carbohydrate digestion and absorption and subsequent glycaemic and insulinaemic responses. It is also possible that any reduced palatability of soaked hay might alter enteric neuroendocrine reflexes during feed prehension, affecting secretory function and motility of the intestine, absorption of carbohydrates and secretion of insulin (Müller and Udén, 2007). Feeding of soaked hay to ponies with conditions such as ID has been recommended previously (Frank et al., 2010) and is further supported by the results of this study.

Contrary to our hypothesis, feeding haylage resulted in greater post-prandial insulinaemic responses than feeding dry hay. Although our hypothesis was based on the typical NSC content of haylage (Müller and Udén, 2007) and the typical forage analysis from the producer of the haylage used in the study (8-11% WSC), the measured NSC content of the haylage used was similar to that of the hay. Haylage with a lower NSC content may have a lower postprandial insulinaemic response, as has been shown for dry hay with a low NSC in healthy Quarter horses and those with polysaccharide storage myopathy (Borgia et al., 2011). However, haylage with similar NSC to dry hay clearly resulted in a higher postprandial

insulinaemic response and its use in ponies with ID instead of soaked hay is not supported by our data.

Analysis of NSC in the current study used wet chemistry analytical methodologies to quantify WSC and starch (NSC = WSC + starch) in forage dry matter. These direct analytical methods were selected over near infrared reflectance spectroscopy (NIRS), on the basis that wet chemistry was more likely to yield exact measures for the presented feedstuffs (Corson et al., 1999). By contrast, analyses provided by the haylage manufacturer and other studies have relied on NIRS analysis, where constituents are derived using prediction models from wet chemistry analysis (Corson et al., 1999). Fermentation of grass results in utilisation of simple sugars and the production of alcohols and simple acids (Müller and Udén, 2007). While these fermentation products remain within the overarching classification of WSC, they are increasingly volatile and inclusion of these simple compounds may be highly method dependent. Furthermore, this study and other laboratories generally use heat drying to constant mass to prepare dry matter samples for further analyses. This pre-treatment might be expected to evaporate simple nutrients thereby excluding energetically important compounds from further analyses. In this study, there was wide variation in the NSC fractions of the two haylage bales analysed by wet chemistry, with WSC values of 21% and 12%, and starch values of 0% and 4% respectively, which reiterates the potential variation in either the nutritional content of different forage samples, or the detection of different sugars and fermentative by-products during analysis, even when from the same batch. These preliminary data emphasise a need for standardisation in analytical methodologies and an improved understanding of the exact chemical compositions of the fractions evaluated.

The difference in insulinaemic response between haylage and the other forages was particularly marked, and causes other than difference in NSC content should be considered. Although short chain- and other rapidly digested carbohydrates are likely to be the primary determinant of the glycaemic and insulinaemic response to forages, other nutrients such as volatile fatty acids and ethanol are present in fermented forages such as haylage and have less well known insulinaemic effects in horses (Argenzio and Hintz, 1970). Further work comparing the insulinaemic effects of fermented and unfermented forages is required and would provide further evidence on which to base feeding strategies.

In this study forages were fed in equal quantities on a dry matter basis. Daily requirements for equine diets are normally calculated according to energy, however, and to provide an isocaloric amount of the different forages used in this study would require 12.7% more

soaked hay or 5.9% more hay, compared to haylage (DM). Although this should be considered when applying these data to clinical cases, the candidate suggest that given the magnitude of the differences in insulinaemic responses between the different forages, the increased amount of soaked hay or dry hay required would have minimal effect.

The animals used in this study were native or native-cross pony breeds, 3-15 years old, mostly overweight (>6/9) BCS and maintained on hay and pasture. Insulinaemic responses to forages in other groups of equids might be different.

Differences in insulinaemic responses to different forages were greater than differences in glycaemic responses. Other studies have also found that the insulinaemic response more closely reflects the NSC content of the feed than the glycaemic response (Vervuert et al., 2009), and is more representative of metabolic status (Borgia et al., 2011). Insulin secretion by the endocrine pancreas is largely under the control of blood glucose concentration, however other mechanisms are involved. It is possible that differences in protein or volatile fatty acid concentrations of feeds, the enteroinsular axis and the rate of digestion of carbohydrates significantly contribute to the post-prandial insulinaemic responses that cannot be accounted for by glucose alone.

In this study, an OGT was used to define ponies with ID, with conventional, but un-validated cut-offs for the classification of positive or negative. A gold standard diagnostic test for ID has not been established, in part due to its heterogenous nature meaning that different elements of ID are evaluated by different tests (Bertin and de Laat, 2017). In several species, including the horse, that enteral glucose results in a greater insulin response compared to an equivalent, isoglycaemic intravenous dose (Duhlmeier et al., 2001). The enteroinsular axis is likely to have a significant role in the insulinaemic response to feed, estimated to represent 25% of pancreatic secretion in the horse (de Laat et al., 2016); hence the OGT was chosen to stratify horses in this study. For convenience, basal and CGIT insulin concentrations were used for pre-study screening to ensure a wide range of ID was included. The dose and type of carbohydrate used in oral tests, and whether it should be administered as part of a feed or as simple sugars requires further study.

In conclusion, feeding soaked hay results in a lower glycaemic and insulinaemic response compared to an equal amount on a dry matter basis of unsoaked hay from the same batch. ID ponies show greater insulinaemic response to all forages compared to non-ID ponies; thus, feeding diets that minimise insulinaemic response is likely to be particularly important in

these animals. Feeding haylage can result in marked post-prandial hyperinsulinaemia, particularly in ponies with ID. NSC content is variable between forages and should be measured when determining dietary plans.

## 5.6 Manufacturers' Addresses

[1] Leominister UK

[2] Milton Keynes, UK

[3] Bickerstaffe, Lancs, UK

[4] Selby, Yorkshire, UK

[5] Camberley, Surrey, UK

[6] Dorset, UK

[7] Yellow Springs, OH, USA

[8] New York, USA

# Chapter 6: Investigation of Glucagon-like peptide-1 response to six oral carbohydrates in ponies

# 6.1 Abstract

Further data on the role of incretins in the pathophysiology of insulin dysregulation (ID) may facilitate advances in its diagnosis and treatment. Glucagon-like peptide-1 (GLP-1) is the principal incretin in horses and this study aimed to describe its concentration in response to three preserved forages and four dynamic tests for ID in ponies. Twelve adult ponies of mixed ID status were given a meal of hay, soaked hay or haylage, an in-feed oral glucose test (OGT), oral sugar (Karo syrup) test (OST), proprietary cereal test (WEET) or a combined glucose-insulin tolerance test (CGIT) each week in a randomised cross-over study design. Glucose, insulin and GLP-1 concentrations were measured before and following each of these interventions. Ponies were designated ID or non-ID twice, according to the OGT result and the CGIT result.

All interventions apart from the CGIT provoked a GLP-1 response within 30 min, and for forages, the GLP-1 concentration peak preceded the insulin concentration peak. The OGT and WEET interventions, which contained the greatest dose of non-structural carbohydrate (1.06 and 1 g/kg BW, respectively), resulted in a greater area under the curve (AUC) for GLP-1 compared to all other interventions ($P < 0.001$). No difference in GLP-1 response was detected between ID-positive and ID-negative groups, despite there being strong positive correlation (r (95% CI)) between GLP-1 and insulin concentrations measured at individual times points (0.67 (0.62 – 0.71); $P < 0.001$) and as AUC (0.66 (0.49 – 0.79), $P < 0.001$). There was no significant GLP-1 response to the CGIT. Preserved forages and the oral dynamic tests resulted in a rapid GLP-1 response. These data do not support of the use of GLP-1 as an adjunctive diagnostic test for ID, as defined by conventional intravenous or oral dynamic tests.

## 6.2 Introduction

A causal association between hyperinsulinaemia and laminitis has been established through experimental and clinical trials (Asplin et al., 2007, Treiber et al., 2006). The abnormalities of insulin metabolism which lead to hyperinsulinaemic laminitis are collectively termed insulin dysregulation (ID) and include tissue insulin resistance and excessive post-prandial hyperinsulinaemia (Frank and Tadros, 2014). Post prandial hyperinsulinaemia is due to gastrointestinal potentiation of pancreatic insulin secretion and part of what is called the enteroinsular axis (EIA) (de Graaf-Roelfsema, 2014). In horses and humans, the EIA is evidenced by measuring a greater insulinaemic response to orally administered glucose compared to an isoglycaemic dose administered intravenously (de Laat et al., 2016, Hampton et al., 1986, Duhlmeier et al., 2001). In insulin dysregulated horses the EIA response to feeding can be excessive or exaggerated (Durham et al., 2019, Frank and Tadros, 2014).

The EIA response is mediated by hormones called incretins which include glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). GLP-1 forms the major component of the EIA in horses, estimated in one study to increase pancreatic insulin response by 23% compared to glucose alone (de Laat et al., 2016). Other effects of GLP-1 in humans include inhibition of glucagon secretion, appetite and gastric motility (Campbell and Drucker, 2013). These additional effects have not been investigated in horses; however, it has been demonstrated that the GLP-1 receptor (GLP-1R) is distributed across a wide range of tissues (Kheder et al., 2018). Pharmacological manipulation of GLP-1 metabolism and the GLP-1R are widely used to augment insulin secretion and cause other, extra-pancreatic effects in humans with type-2 diabetes (Nauck, 2016). For the management of horses with ID, a reduction in pancreatic insulin secretion could be beneficial, and recently this occurred following application of a GLP-1R antagonist to pancreatic islets *in vitro* (Kheder et al., 2018). Further evaluation of the GLP-1 responses to different feeds and between ID and non-ID horses will help determine whether GLP-1R has potential as a therapeutic target in horses.

Measurement of post-prandial hyperinsulinaemia following oral carbohydrate administration, or basal insulin during consumption of preserved forage or pasture is advocated for detection of an exaggerated response and diagnosis of ID (Durham et al., 2019, Frank et al., 2020). Oral tests, compared to intravenous tests, include the effect of the EIA as well as hyperinsulinaemia due to insulin resistance and may be more sensitive at detecting ID (Durham et al., 2019, Frank and Tadros, 2014). There is, however, suboptimal repeatability and agreement between different oral carbohydrate and basal tests (Borgia et al., 2011, de

Laat and Sillence, 2017, Knowles et al., 2016) and cut-offs or diagnostic thresholds have not been well described.

GLP-1 concentration could offer useful additional diagnostic information for the assessment of insulinaemic responses to feeding and ID. The relationship between GLP-1 and insulin concentrations is incompletely described. Insulin and GLP-1 concentrations were positively correlated following NSC ingestion (Bamford et al., 2015, de Laat et al., 2016) and grazing (Fitzgerald et al., 2019b), whereas there was no relationship following an oral sugar test (OST) (Frank and Walsh, 2017) and high NSC feed (Meier et al., 2020). Evidence for an association between GLP-1 concentration and ID status is also mixed (Bamford et al., 2015, de Laat et al., 2016, Chameroy et al., 2016, Fitzgerald et al., 2019b). Data are lacking on GLP-1 concentration following consumption of preserved forages. Further work is required to help understand the contribution of the enteroinsular axis to ID, and its potential as a target for diagnosis or therapy.

This study describes GLP-1 response to six different forms of oral carbohydrate and a combined glucose insulin tolerance test (CGIT) in a group of twelve ponies of varying ID status. Insulin and glucose responses to these interventions have been described in earlier chapters, and here we use these data to examine their relationship to GLP-1. We hypothesised that GLP-1 response would be associated with NSC content of the feed, and that it would be greater in ID-positive compared with ID-negative ponies. Further, we hypothesised there would be minimal GLP-1 response to intravenously administered glucose, and that insulin and glucose concentrations would be correlated with GLP-1 concentration following oral carbohydrate.

## 6.3 Materials and methods

The study was conducted under the Animals (Scientific Procedures) Act 1986 (project licence PPL 40/3715).

### 6.3.1 *Animals*

Twelve adult ponies were selected for the study according to criteria described in Chapters 4 and 5. Each week during the habituation period and throughout the study, bodyweight and girth and belly circumferences (cm) were measured, body condition score (BCS) (Kohnke, 1992) and cresty neck score (CNS) (Carter et al., 2009a) were estimated, and a clinical examination performed. The same group of 12 ponies as in Chapters 4 and 5 was used, with

the sample size calculated for the study in Chapter 4. No preliminary data on GLP-1 concentrations existed to enable sample size or prospective power calculations.

### 6.3.2 *Housing and feed*

Ponies were individually stabled on wood shavings for 22 h/day and turned out with a closed grazing muzzle (Shires Equestrian[1]) into a 0.3 Ha paddock for free exercise for 2 h/day. Daily feed intake was standardised at 2% body weight (BW) fresh weight using same batch, dry meadow hay provided as two daily meals (0.5% BW at 12:00 after turnout and 1.5% BW at 17:00). A proprietary feed balancer (Lite Balancer, Spillers Feeds[2]) was provided (200 g/pony/day).

### 6.3.3 *Study protocol*

The study had a 7-way, randomised (www.randomizer.com) cross-over design so each pony received each intervention on one occasion, and the order for each pony was randomised. Each week over the 7-week study period a catheter was placed in the jugular vein. That evening, hay provision was restricted (1% BW fed at 17:00) to ensure complete consumption by midnight. The following morning, each pony had either a single forage meal (0.25% BW DM) of hay, soaked hay or haylage, an oral glucose test (OGT), OST, proprietary breakfast cereal (WEET) or CGIT. Time to complete all meals was recorded, and any residual OGT or WEET removed and weighed after 40 min. The catheter was removed after the last blood sample and ponies returned to the regular study routine.

### *6.3.4 Interventions*

Details of the forages, including nutritional analysis are provided in Chapter 5, and details of OGT, OST, WEET and CGIT protocols are provided in Chapter 4. The NSC provided in each test was calculated as the sum of starch and water soluble carbohydrate from nutritional analysis for hay, soaked hay and haylage (Chapter 5), manufacturer supplied nutritional analysis for Happy Hoof[2] (used in OGT) and WEET and previously published analysis of the corn syrup (Karo Light[3]) (Jocelyn et al., 2018, Schuver et al., 2014) used in OST (Table 1).

A baseline blood sample (t=0) was obtained before all interventions, and then 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135, 150 and 180 min after glucose and insulin administration for the CGIT and 30, 60, 90, 120, 150, 180, 240 and 300 min following the other interventions.

**Table 6.1:** Non-structural carbohydrate (g/kg BW) concentration of the different interventions as determined by wet chemistry nutritional analysis or published data.

| Intervention | Non-structural carbohydrate (g/kg BW) |
|---|---|
| Hay | 0.4 |
| Soaked Hay | 0.27 |
| Haylage | 0.46 |
| OGT | 1.06 |
| OST | 0.14 (Jocelyn) 0.15 (Schuver) |
| WEET | 1 |
| CGIT | 0.15 (Intravenous) |

### *6.3.5 Blood processing and analysis*

For all time points, blood was collected in fluoride oxalate tubes, placed in ice and then whole blood glucose concentration was measured in duplicate using a glucose oxidase enzymatic method (YSI 2300 STAT[4]) within 8 h. Blood obtained 0, 25, 45, 60, 75, 90, 120, 150 and 180 min after CGIT and at all time points following the other interventions was also analysed for active GLP-1 and insulin concentrations. For GLP-1, 2ml of blood was immediately placed into EDTA tubes to which 40 µL of a proprietary mixture of dipeptidyl peptidase-4 (DPP-4) inhibitors (DPP-IV inhibitor, Millipore[5]) had already been added, and stored immediately in ice. Within 5h tubes were centrifuged at 2000 *g* for 10 min at 4°C, and the plasma harvested and stored at -80°C until analysis. GLP-1 concentration was measured in duplicate using a human active GLP-1 enzyme-linked immunosorbent assay (ELISA, Millipore[5]), previously validated for use in horses (Chameroy et al., 2010). Mean intra- and interassay coefficients of variation (CVs) were 8.0% and 15.6%, respectively. A delay in the supply of DPP-4 inhibitor meant that 66 out of 108 GLP-1 samples in the second week were missing. Blood for insulin analysis was placed in plain tubes and left at room temperature to clot for 1-2 hours, after which it was centrifuged at 2000 *g* for 10 min and the serum separated and stored at 5°C until analysis within 8 h of collection. Insulin concentration was measured using a previously validated chemiluminescent assay (Immulite 2000, Siemens[6]) with intra- and interassay CVs of 1.8-2.4% and 3.0-7.1% respectively (Chapter 3).

### *6.3.6 Data analysis*

Normality of data was assessed using the Shapiro Wilk test and assessment of histograms. Where required, $Log_{10}$ transformation was performed after addition of 0.5 x the minimum value to avoid $log_{10}$ of zero. Basal (fasting) GLP-1 concentration was taken as the t = 0 min sample for each intervention. Time of maximal GLP-1 ($Tmax_{GLP-1}$), insulin ($Tmax_{insulin}$) and glucose ($Tmax_{glucose}$) concentrations were the time point after feeding with the greatest concentration. Obesity was defined as a BCS ≥ 7/9 and binary ID status determined for each horse according to previously published cut-offs for the OGT and CGIT (Chapter 4).

Differences in basal GLP-1 concentration according to ID status were evaluated using a Mann Whitney-U test. Relationships between GLP-1 and glucose and insulin concentrations for all sampling points following oral carbohydrate interventions (Hay, Soaked hay, Haylage, OGT, OST and WEET) was evaluated using Spearman's rank correlation coefficient. A comparison of Tmax between different interventions was performed visually using box plots.

Area under the curve for GLP1 ($AUC_{GLP-1}$) was calculated using the trapezoid method with zero as the baseline. Relationships between $Log_{10}AUC_{GLP-1}$ and $Log_{10}AUC_{glucose}$ and $Log_{10}AUC_{insulin}$ following oral carbohydrates were evaluated using Pearson's correlation coefficient.

A 2-level linear regression model (test, horse) was established with $Log_{10}AUC_{GLP1}$ as the outcome variable, and within-horse effects due to cross over design accounted for by incorporation of horse as a random intercept term. The effect of the interventions (Hay, Soaked hay, Haylage, OGT, OST, WEET), obesity and ID status according to OGT and according to CGIT were assessed by entering variables in the linear regression model. An interaction term of intervention*ID status was assessed to determine any differing effect of the intervention in ID positive or negative ponies. For each intervention, the mean (± s.e.) $Log_{10}$ $AUC_{GLP-1}$ was estimated after adjusting for clustering at the horse level by reporting the intercept. Model diagnostics included evaluation of the normality of the residuals using normal probability plots.

Statistical analysis was performed using commercial software (SPSS 27, IBM[7] and MLwIN 3.05, Centre for multilevel modelling[8]). Significance (2-sided) was defined as $P < 0.05$.

### *6.3.7 Dates and location of the study and the candidate's role*

The cross over study took place in 2014 at the University of Liverpool. Analysis of samples was performed 2014 – 2016. The candidate contributed to study design and organised and performed data collection and sample analysis with assistance from technicians. He performed data analysis and prepared this chapter of the thesis.

## 6.4 Results

### *6.4.1 Animals.*

All ponies were mixed, native British breeds. There were 11 mares and one gelding, aged (mean ± SD) 9.1 ± 3.4 years and with a body weight of 280 ± 49 kg. The median BCS was 7.2 (IQR 6 – 7.7) out of 9, with a range of 4.2–8.5. There was no significant difference in mean body weight, girth or belly circumference, CNS or BCS at the time the different interventions were performed, or between ID positive and negative groups. Over the 6–week study period, mean body weight increased from 278 ± 51.2 kg to 284 ± 52.6 kg ($P$ = 0.001).

The OGT meal was entirely consumed by 9/12 ponies, taking (mean ± SD) 23.4 ± 10.7 min. Three ponies consumed between 85% and 95% of the OGT meal which was considered an insignificant reduction. The WEET meal was entirely consumed by 5 ponies, taking 21.8 ± 13.3 min. Six ponies consumed between 56% and 71% of the meal and were included in data analysis. One pony consumed only 34% of the WEET was excluded from further analysis of WEET data. The entire hay, soaked hay and haylage meals were consumed by all ponies. All interventions were well tolerated with no adverse effects seen.

### *6.4.2 GLP-1 concentration*

In the second week of the study 66/108 GLP-1 samples were missing due to delay in the supply of DPP-IV inhibitor, meaning that over the whole study 690/756 GLP-1 samples were included in analysis.

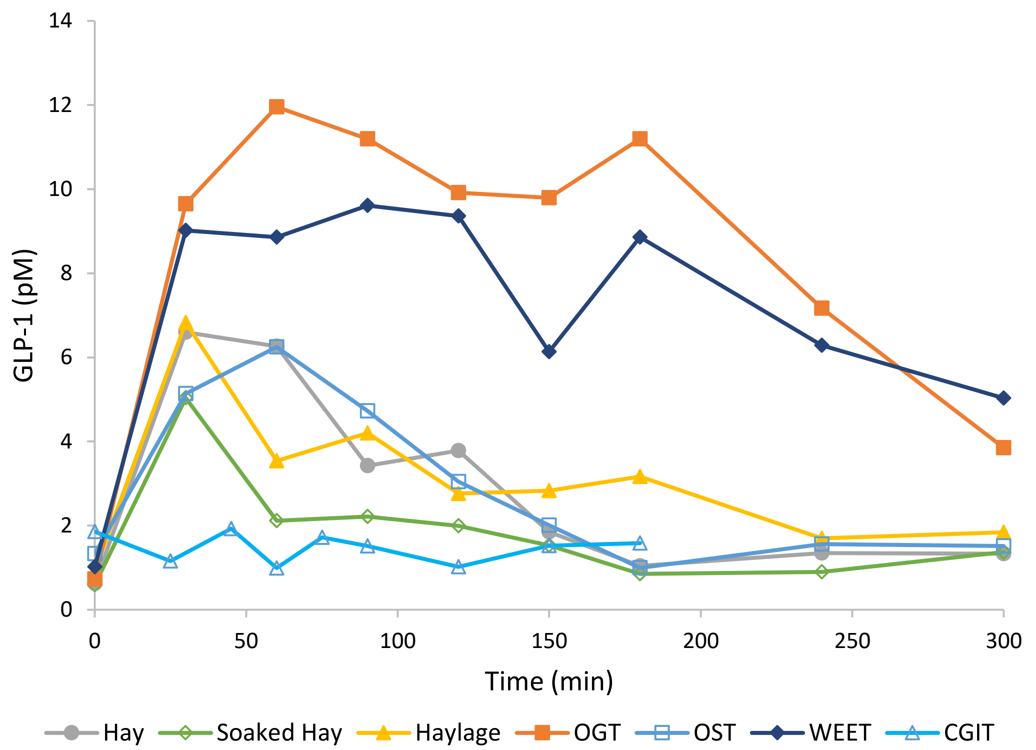
Mean plasma GLP-1, glucose and insulin concentrations following the different interventions are shown in Fig. 1. In comparison to the other interventions, OGT and WEET resulted in greater GLP-1 concentrations which remained increased throughout the 300 min testing period. The other oral carbohydrates interventions resulted in a lower, earlier peak in GLP-1

concentration which reduced to close to basal concentration before 300 min (Figs. 1 and 2). When compared to median $Tmax_{insulin}$ and $Tmax_{Glucose}$, $Tmax_{GLP-1}$ was earlier for all forage interventions, equal for OST and later for WEET (Fig. 2). For all oral interventions there was an early rise in GLP-1 concentration from 0 to 30 min, and then, apart from OST, a second peak after 90 – 180 min (Fig. 1). There was no discernible increase in mean GLP-1 concentration in response to the CGIT.

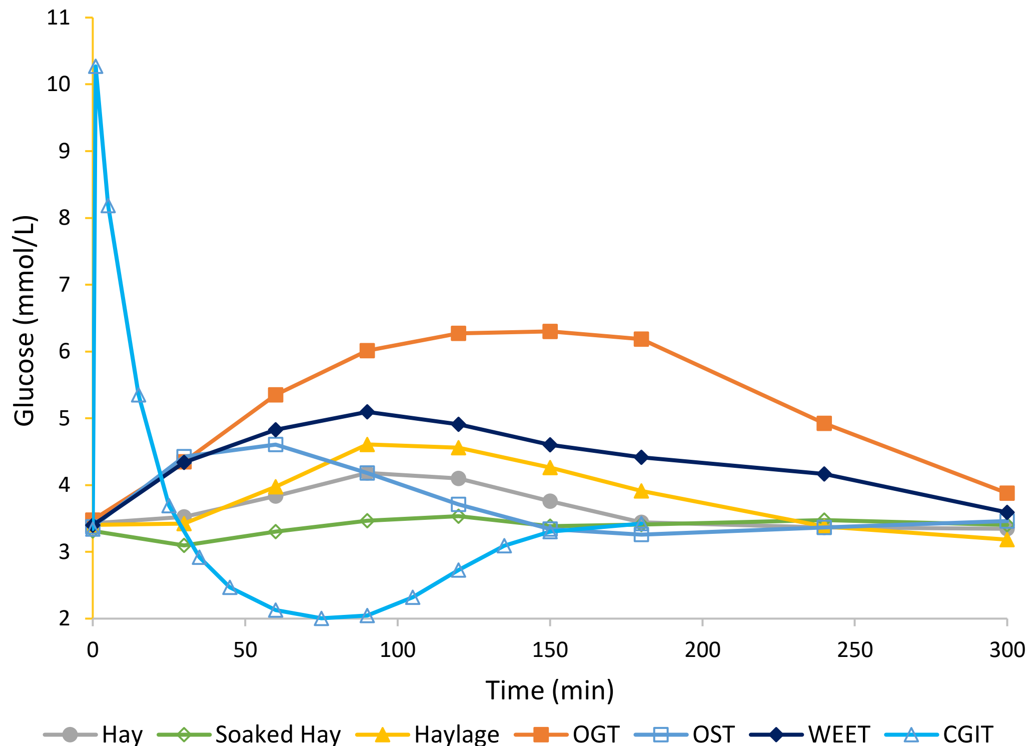
In relation to ID status, there was no difference in median (IQR) basal GLP-1 between CGIT-positive (0.91 pmol/L (0.03 – 1.80)) and CGIT-negative (0.69 pmol/L (0 - 1.45)), or between OGT-positive (0.72 pmol/L (0.14 – 1.48)) and OGT-negative (0.75 pmol/L (0 - 1.55)) animals.

There was a strong correlation ($r_s$ (95% CI)) between GLP-1 and insulin (0.67 (0.62 – 0.71); $P < 0.001$) concentrations and a moderate correlation between GLP-1 and glucose (0.52 (0.46 – 0.58); $P < 0.001$) concentrations.

A




B



C

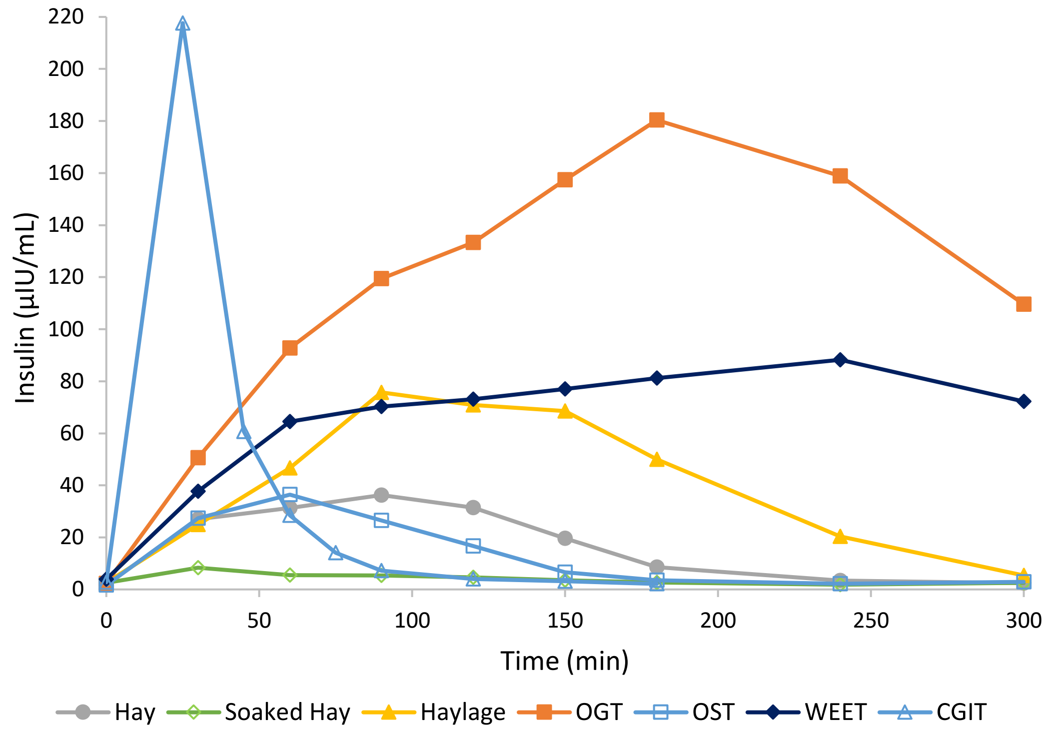



**Fig. 6.1:** Mean glucagon-like peptide-1 concentration (pmol/L) following hay (n = 11), soaked hay (n = 9), haylage (n = 11), oral glucose test (n = 11), oral sugar test (n = 10), Weetabix test (n = 9) and combined glucose-insulin tolerance test (n = 11) (A); Mean Glucose (B) and insulin (C) concentration following the same seven interventions (all n = 12).

### *6.4.3 GLP-1 area under the curve*

The missing GLP-1 concentration data resulted in an inability to calculate $AUC_{GLP-1}$ for 12/84 interventions: 1 x Hay, 3 x Soaked hay, 1 x Haylage, 1 x OGT, 2 x OST, 3 x WEET (including the pony excluded for inadequate consumption) and 1 x CGIT.

There was a strong positive correlation (r (95% CI)) between $Log_{10}AUC_{GLP-1}$ and $Log_{10}AUC_{insulin}$ (0.66 (0.49 – 0.79), $P < 0.001$) and a moderate correlation between $Log_{10}AUC_{GLP-1}$ and $Log_{10}AUC_{glucose}$ (0.58 (0.39 – 0.73), $P < 0.001$).

The multilevel linear regression model showed there was a significant effect of intervention on $Log_{10}AUC_{GLP-1}$ ($P < 0.001$). There was no significant effect of obesity, ID status (as

determined by CGIT or OGT) (Fig 3) or of the interaction terms tested. The predicted mean $Log_{10}AUC_{GLP-1}$ from the regression model, adjusted for within-horse clustering is shown for each intervention in Table 2. $Log_{10}AUC_{GLP-1}$ was significantly greater after OGT and WEET compared to all other interventions (*P* < 0.001). Haylage and OST caused a greater response than soaked hay (*P* = 0.02 and 0.046, respectively), and $Log_{10}AUC_{GLP-1}$ for CGIT was less than all other interventions ((*P* < 0.001). Within-horse variance (s.e.) was 0.024 (0.013). Normal probability plots of standardised residuals showed that the assumption of normality was reasonable (Figs 4 and 5).

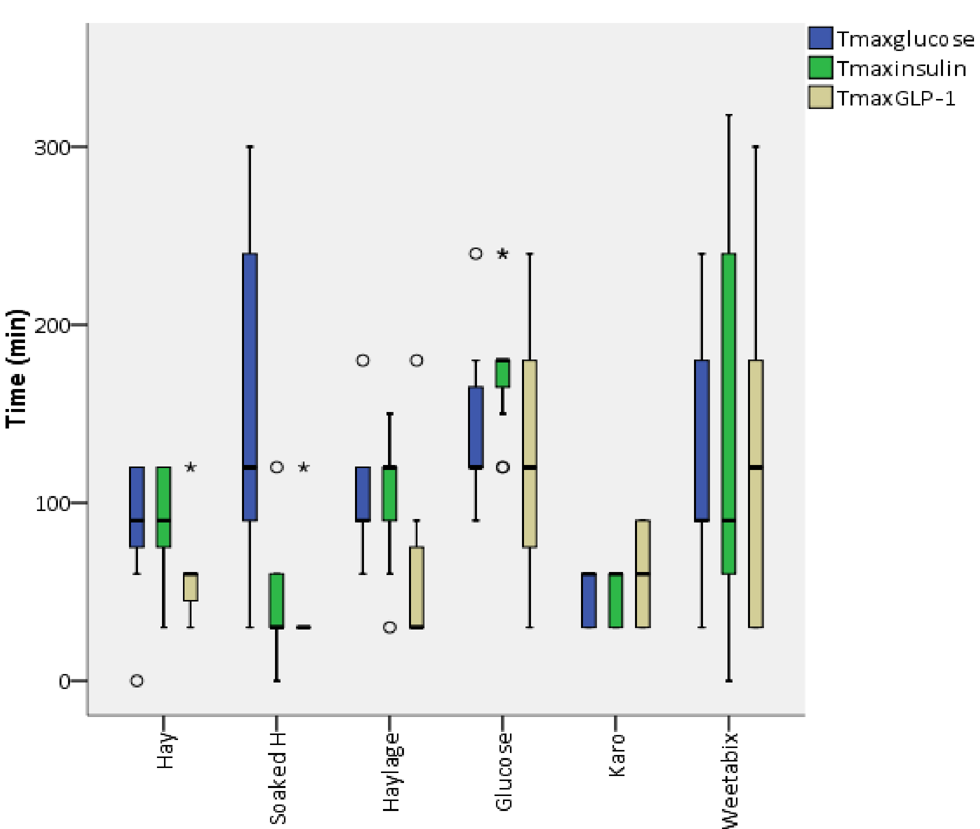



**Fig. 6.2:** Box plot of time of maximal GLP-1 ($Tmax_{GLP-1}$), insulin ($Tmax_{insulin}$) and glucose ($Tmax_{glucose}$) concentrations following hay (n = 11), soaked hay (n = 9), haylage (n = 11), oral glucose test (n = 11), oral sugar test (n = 10) and Weetabix test (n = 9). * = outliers >3 times IQR from the end of a box; o = outliers 1.5 – 3 times IQR from end of the box.

**Table 6.2:** Mean and s.e. $Log_{10}$ Area under the curve for glucagon-like peptide-1 (min*pmol/L) following seven different interventions. OGT; oral glucose test, OST; oral sugar test, WEET; Weetabix test, CGIT; combined glucose-insulin tolerance test (CGIT). *All interventions were measured over 300 min except CGIT which was measured over 180 min. $^{a-f}$ Values with the same superscript letter are significantly different.

| **Intervention** | **Mean $Log_{10}$ $AUC_{GLP-1}$** | **S.E.** | ***P* Value** | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **Hay Test 1** | **Soaked hay Test 2** | **Haylage Test 3** | **OGT Test 4** | **OST Test 5** | **WEET Test 6** |
| Hay | $2.87^{ac}$ | 0.078 | - | - | - | - | - | - |
| Soaked hay | $2.70^{bd}$ | 0.084 | 0.085 | - | - | - | - | - |
| Haylage | $2.93^{bf}$ | 0.078 | 0.484 | 0.017 | - | - | - | - |
| OGT | $3.37^{abe}$ | 0.078 | <0.001 | <0.001 | <0.001 | - | - | - |
| OST | $2.90^{de}$ | 0.081 | 0.736 | 0.046 | 0.731 | <0.001 | - | - |
| WEET | $3.27^{cdf}$ | 0.084 | <0.001 | <0.001 | <0.001 | 0.296 | <0.001 | - |
| CGIT* | $2.21^{abd}$ * | 0.078 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

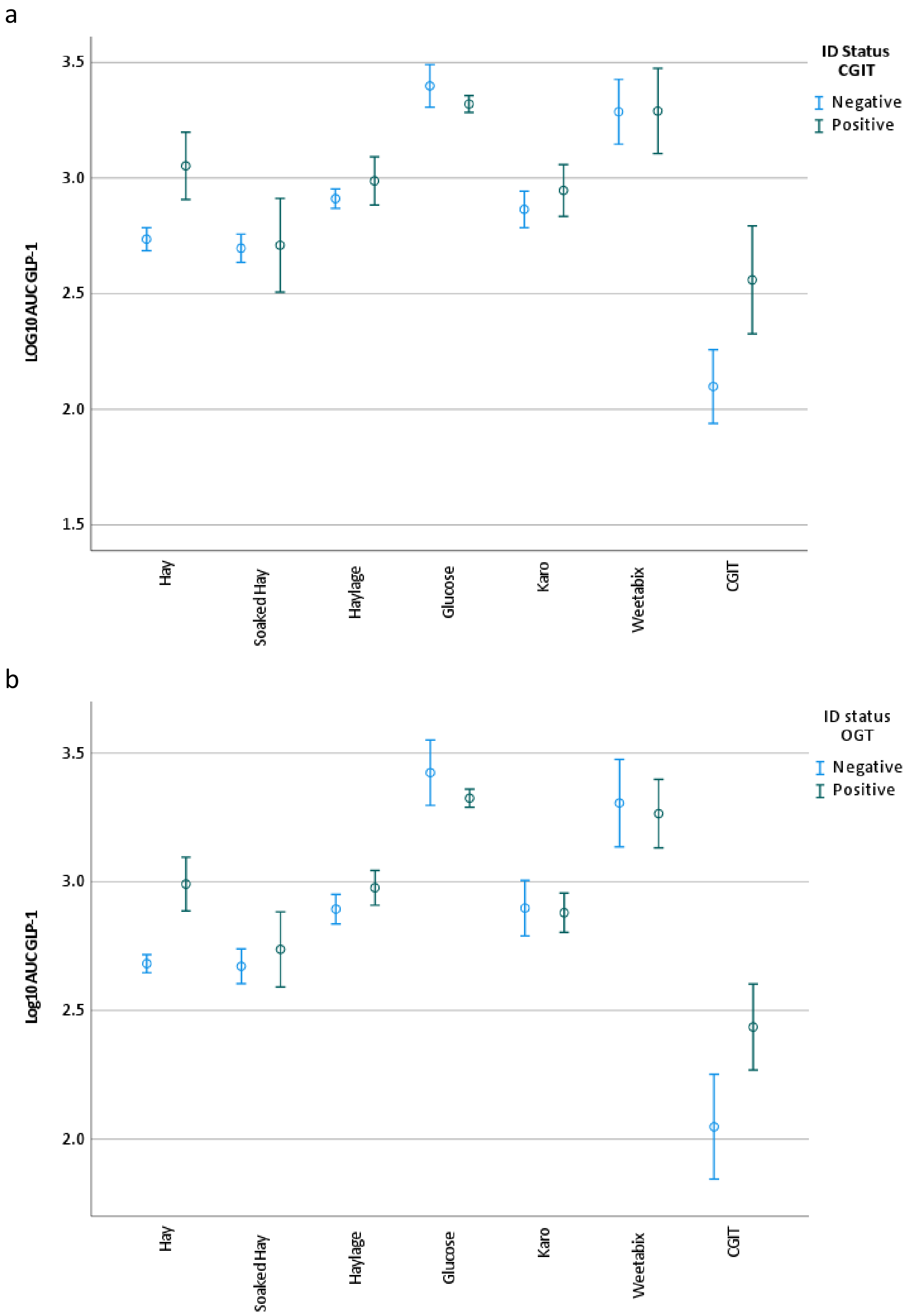



**Fig. 6.3:** Mean +/- s.e. $Log_{10}$ Area under the curve for GLP-1 ($Log_{10}$ $AUC_{GLP-1}$, min*pmol/L) following seven different interventions in CGIT positive (n = 4, green) and negative (n = 8, blue) animals (A), and OGT positive (n = 6, blue) and negative (n = 6, green) animals (B).

Area under the curve was calculated using GLP-1 measurements over 300 min for all interventions except CGIT which was only measured over 180 min. Within-horse variance (s.e.) was 0.024 (0.013)

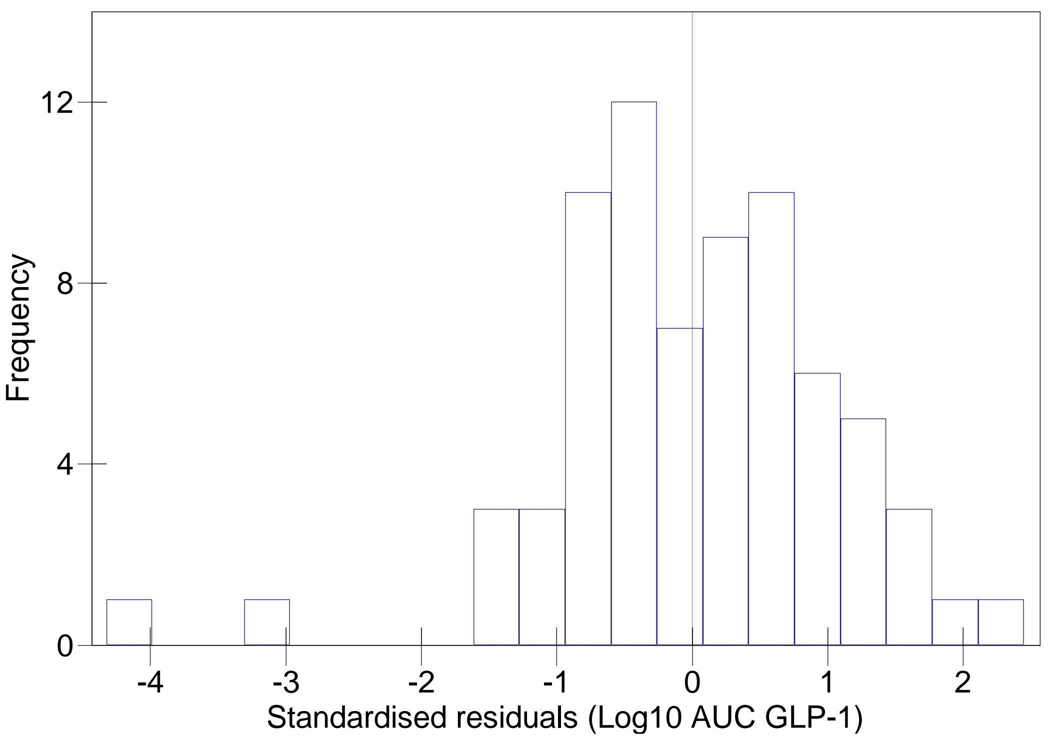



**Fig. 6.4:** Histogram of standardised residuals of the 2-level linear regression model with $Log_{10}$ $AUC_{GLP-1}$.as outcome.

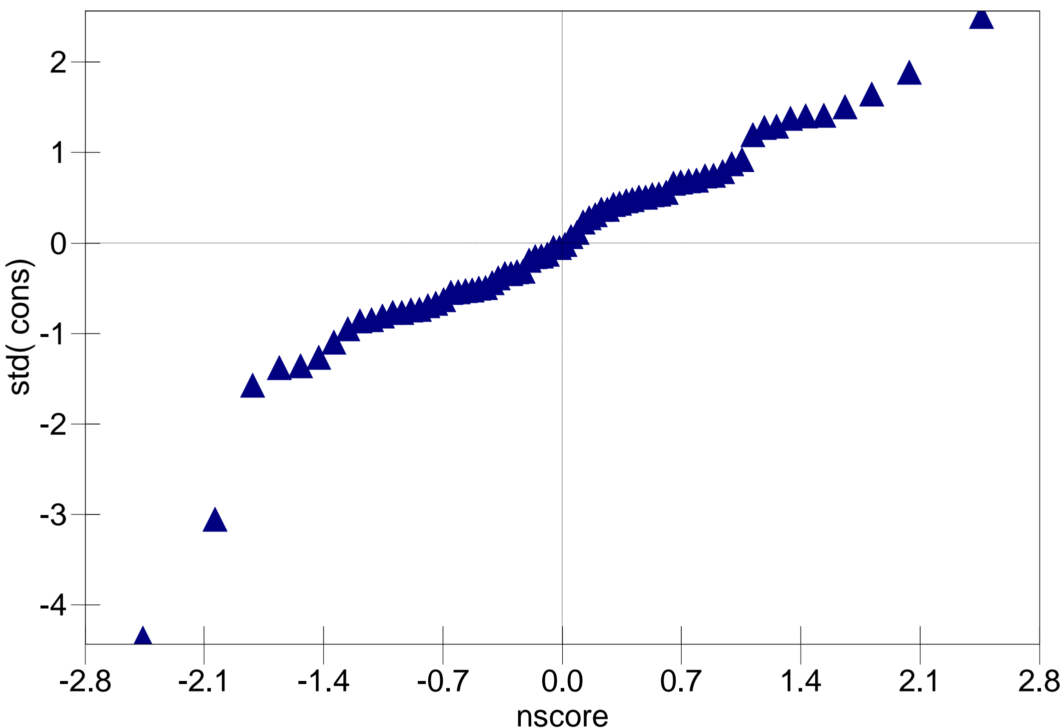



**Fig. 6.5:** Normal probability plot of standardised residuals against a normally scores.

## 6.5 Discussion

This study provides further evidence of a functional enteroinsular axis in ponies and a detectable GLP-1 response to a variety of feeds, including some with a low NSC content. It was performed in ponies as these breeds are particularly susceptible to endocrinopathic laminitis (Jeffcott et al., 1986, Pollard et al., 2019a).

This is the first study to evaluate GLP-1 response to preserved forages, including soaked and unsoaked hay from the same bale. Soaking hay in water reduces its NSC content (Mack et al., 2014), and results in significantly lower post-prandial glycaemic and insulinaemic responses (Chapter 5). There was no significant reduction in GLP-1 concentration following soaked- compared to unsoaked hay identified in this study, indicating that the difference in insulin response is likely to be driven principally by the small but significant difference in the blood glucose response between soaked and unsoaked hay (mean maximum glucose concentration ($Cmax_{glucose}$) 3.7 versus 4.1 mmol/L, respectively, Chapter 5). Haylage ($Cmax_{glucose}$ 4.9mmol/L) resulted in a significantly greater GLP-1 response than soaked, but not unsoaked hay. The inability to discriminate between haylage and hay may be due to the large GLP-1 response to unsoaked hay in ID-positive ponies (Fig. 3). It has been demonstrated in rodents that GLP-1 secretion following oral carbohydrate is dose-dependent (Yoder et al., 2010) and this difference is likely to have been driven, at least in part, by differences in NSC content and availability between the three forages. The fermentation process of haylage also makes it more digestible and available for absorption (Müller, 2012). In Chapter 5 we proposed that the greater serum insulin response following haylage compared to hay and soaked hay could have had causes other than just greater NSC content and availability. Intestinal amino acids and fats result in GLP-1 secretion in humans (Diakogiannaki et al., 2012), and the greater crude protein concentration in the haylage fed might have influenced the GLP-1 response. Additionally, it is possible that GLP-1 secretion is affected by the fermentation products such as volatile fatty acids and ethanol that are present in haylage but not hay, but based on the lack of significant difference in GLP1 between hay and haylage this theory is unlikely.

Similar to the forages, GLP-1 responses appeared to be related to the NSC content in the oral carbohydrate tests. The main differences in this study were the greater GLP-1 responses to both OGT and WEET, which were fed at approximately 1g/kg NSC compared to less than half of this in the hay and haylage (approx. 0.4 g/kg) and even lower in the OST. The GLP-1, glucose and insulin responses to the OST were greater than expected considering its very low NSC content compared to the other interventions (Jocelyn et al., 2018, Schuver et al., 2014).

Higher digestibility and availability of the NSC in corn syrup, the bolus dose or variability in the NSC content of corn syrup in the OST could account for this. There was no discernible GLP-1 response to intravenously administered glucose, as has been reported in other studies for GLP-1 (de Laat et al., 2016) and GIP (Duhlmeier et al., 2001). The exogenous insulin bolus administered as part of the CGIT shortens the period of hyperglycaemia and could have altered the GLP-1 response. In this study blood samples following the CGIT were obtained over 180 min compared to 300 min for the other interventions, which should be considered when comparing the areas under the curve.

Contrary to our hypothesis, GLP-1 was unable to differentiate ID and non-ID ponies, as identified by the CGIT and OGT. This is despite the ID group showing clearly exaggerated hyperinsulinaemic responses to all oral interventions (Chapters 4 and 5) and the strong positive correlation identified between insulin and GLP-1 concentrations at individual time points and between $AUC_{insulin}$ and $AUC_{GLP-1}$. In Fig. 3, the GLP-1 response following hay appeared to be greater in ID-positive compared to ID-negative ponies. This difference was not detected statistically, however, possibly because the study had insufficient power. Other studies have found variable associations between ID status and GLP-1 concentration, with one study even showing a negative correlation between GLP-1 and insulin and a lower GLP-1 concentrations in the ID group, when measured at two time points following an OST (Frank and Walsh, 2017). GLP-1 concentration following an OGT did not predict the development of laminitis in a group of ponies subsequently exposed to a high NSC dietary challenge (Meier et al., 2020). Similar to this study, others have also shown a positive correlation between insulin and GLP-1 concentrations (Bamford et al., 2015, de Laat et al., 2016). The metabolic derangements leading to hyperinsulinaemia and laminitis are complex and likely vary between individual horses (Frank and Tadros, 2014). Intravenous tests such as the CGIT assess insulin resistance only, and so fail to capture the effect of the EIA. Oral tests such as the OGT include the contribution of the EIA to insulin concentration, but post-prandial glucose absorption has a greater insulinaemic effect than incretins (de Laat et al., 2016). For these reasons, use of conventional tests for ID might not be an appropriate way to dichotomise horses for assessment of GLP-1 concentration as a diagnostic test.

Median Tmax or Tmax of the mean concentration of GLP-1 was earlier than, or equal to those of insulin and glucose, which were more closely aligned (Figs. 1 and 2). This is consistent with findings in other equine studies (de Laat et al., 2016, Fitzgerald et al., 2019b), which showed that for Tmax, GLP-1 preceded glucose and insulin following oral carbohydrate. The secretion of GLP-1 is biphasic in rats and humans, the first phase occurring within 10-15 min and

predominantly mediated by neuroendocrine mechanisms rather than direct contact of nutrients on the secretory L-cells which are mostly located in the distal small intestine (Baggio and Drucker, 2007). These mechanisms could also account for the similar timing of the early GLP-1 peak between the different interventions, in contrast to the peaks of glucose and insulin which were more variable. Although not present for all interventions, a biphasic GLP-1 response was discernible for most, as reported in humans (Herrmann et al., 1995) and horses (de Laat et al., 2016).

GLP-1 was the only incretin measured in this study, for financial reasons and because it has been shown to have a greater effect on post-prandial insulin secretion compared to GIP (de Laat et al., 2016). Another insulinotropic peptide, glucagon-like peptide 2 (GLP-2) has been identified in horses (de Laat et al., 2018) and measurement of this and GIP together with GLP-1 could offer a greater insight into the EIA and its association with ID. Further work is required to understand the role GLP-1 and other incretins could have in diagnosis and management of ID. Difficulties in sample handling are likely to limit the diagnostic use of GLP-1 in clinical practice and the extrapancreatic effects of GLP-1R antagonism and possible association with hyperglycaemia require further investigation before therapeutic use can be advised.

There were missing samples in this study, which may have weakened the power of the study to detect differences and to detect significant interactions between ID and the intervention. Due to missing values, multilevel modelling adjusted for repeated measures within horses was used for analysis instead of repeated measures ANOVA. Multiple imputation was not used as the data were missing completely at random, it was only the dependent variable missing, and it was a small and non-normally distributed dataset. A prospective sample size calculation was not performed for this study, as data relating to likely GLP-1 responses, and clinically significant differences were lacking. This would have resulted in the study being more susceptible to insufficient statistical power and type-2 error. Pony breeds were chosen for this study as they are known to be at increased risk of hyperinsulinaemic laminitis and are commonly presented for veterinary investigation. Ponies and Andalusian horses had a greater GLP-1 response to feed compared to standardbreds in a different study (Bamford et al., 2015) and the data presented here might not be applicable to other breeds. The interassay CV calculated for the GLP-1 assay used in this study was 15.6%, and this may have been a source of measurement error, likely caused by operator error or imprecision of the equipment used during assays. Although >10%, the interassay CV was close to that reported in the assay characteristics from the manufacturer, which gave interassay CVs of 13% and 10% for mean concentrations of 4.5pmol/L and 21.4pmol/L, respectively (Millipore, 2012).

In summary, consumption of preserved forages and the oral dynamic tests resulted in a GLP-1 response within 30 minutes. Different carbohydrate feeds altered GLP-1 responses, but soaking hay did not cause a significant reduction. Contrary to our hypothesis, no association between GLP-1 response and ID status was detected, indicating GLP-1 has a limited use as an adjunctive test for ID, as defined by the OGT and CGIT. There was a strong positive correlation between GLP-1 and insulin responses, however, and further work is warranted to determine if GLP-1 is a suitable diagnostic and therapeutic target for ID in horses.

## 6.6 Manufacturers' Addresses

[1] Leominister UK

[2] Milton Keynes, UK

[3] ACH Food Companies Inc. Cordova, TN, USA

[4] Yellow Springs, OH, USA

[5] Darmstadt, Germany

[6] Camberley, Surrey, UK

[7] New York, USA

[8] University of Bristol, UK

# Chapter 7: Equine Metabolic Syndrome in UK native ponies and cobs is highly prevalent with modifiable risk factors

A modified version of this chapter has been published as a paper

(See Supplementary information)

Carslake, H.B., Pinchbeck, G.L., McGowan, C.M. (2021)

Equine Metabolic Syndrome in UK native ponies and cobs is highly prevalent with modifiable risk factors

*Equine Veterinary Journal* 53, 923 - 934

## 7.1 Abstract

The epidemiology of Equine Metabolic Syndrome (EMS) is poorly described. The objectives of this study were to estimate the prevalence of EMS in native UK ponies and cobs in England and Wales and identify associated risk factors, using a cross sectional study. Breeders registered with UK native pony breed societies and registered riding schools and livery yards within a set radius were invited to participate. All native UK ponies and cobs aged 3-14 years and not diagnosed or being treated for conditions likely to affect insulin regulation at participating premises were eligible. Animals underwent a clinical examination and an oral glucose test while their owner or keeper completed a questionnaire by face-to-face interview. Data were analysed by multilevel uni- and multivariable modelling using insulin concentration and EMS diagnosis as outcomes.

A total of 354 animals were examined at 64 properties (19 studs, 19 livery yards, 26 riding schools). The overall prevalence of EMS adjusted for clustering within yard was 23.3% (95%CI 17.9–29.8%). Risk factors associated with a diagnosis of EMS included age, being female, more sedentary main activity, obesity, and shorter periods on pasture during the summer. Compared to the Welsh section A, the other Welsh, Connemara and cob breeds all had decreased odds of EMS. Clinical manifestations of hoof growth ring and supraorbital fat scores of 3/3 were more frequent in EMS ponies and animals with a history of laminitis within the last 5 years (9.7%) were 14.4 (95% CI 5.9–35.3) times more likely to be positive for EMS than those without. Results may not be transferable to other breeds or age groups. EMS is highly prevalent in UK native ponies and cobs with modifiable risk factors including obesity and sedentary activities. Modifying risk factors could help reduce the risk of laminitis in susceptible animals.

# 7.2 Introduction

Equine metabolic syndrome (EMS) is a recognised collection of risk factors for endocrinopathic laminitis (Durham et al., 2019). Several metabolic disturbances have been associated with EMS (Carter et al., 2009c, Menzies-Gow et al., 2017, Treiber et al., 2005) but, of these, insulin dysregulation (ID) is now recognised as the defining feature of the condition (Durham et al., 2019).

Early identification of EMS is important, as there are effective management strategies that can be implemented to reduce the likelihood of laminitis developing (Morgan et al., 2016a). This includes both identification of at-risk animals, as well as diagnostic tests to confirm the disease. A definitive diagnosis of EMS requires demonstration of ID, for which a range of basal and dynamic diagnostic tests have been described. Dynamic tests for ID are more sensitive (Kronfeld et al., 2005), but can also be more invasive and time consuming; hence less practical for use as a screening test.

Several studies have investigated the incidence of and risk factors for laminitis (Menzies-Gow et al., 2017, Pollard et al., 2019b, Wylie et al., 2011) and obesity (Robin et al., 2015), but the epidemiology of EMS is poorly described and is generally restricted to single herds (Carter et al., 2009c, Treiber et al., 2005) or epidemiological studies using less sensitive tests for EMS such as basal insulin (Morgan et al., 2014). Anecdotally, native pony and cob breeds are considered particularly susceptible to EMS, and experimental studies have shown mixed breed ponies to be more insulin resistant than Standardbreds (Bamford et al., 2014). More recently, several metabolic traits of EMS were shown to have moderate to high heritability in Welsh ponies (Norton et al., 2019b).

Identification of horse, management and phenotypic risk factors for EMS will improve identification of at-risk animals for further, targeted diagnostic testing, screening for ID or implementation of EMS management based on a presumptive diagnosis. Furthermore, identification of modifiable risk factors will allow more effective management strategies to be employed, ultimately reducing the risk of laminitis. Therefore, the aim of the present study was to estimate the prevalence of EMS in native UK ponies and cobs and their crosses in England and Wales, and to identify associated risk factors and clinical manifestations.

# 7.3 Materials and methods

## *7.3.1 Study sample*

The study population included UK native ponies and their crosses and cobs aged 3-14 years in northwest England and north Wales. Breeders registered with UK native pony breed societies and riding schools and livery yards registered with the British Horse Society[1] or with details available on a local equine website[2] and within a 50km radius of the University of Liverpool, Leahurst for breeders and livery yards, or a 75km radius for riding schools were invited by letter and email to participate in the study. Exclusion criteria were animals aged <3 or >14 years, lactating or in the last trimester of pregnancy, diagnosed with pituitary pars intermedia dysfunction (PPID) or other systemic disease, with laminitic foot pain, or receiving any medication likely to affect insulin regulation. The breed of pony was as reported by the owner. It was requested that all eligible animals at participating premises were included. Using an estimated prevalence of 25% for EMS (Morgan et al., 2014, Pleasant et al., 2013), a sample size of 289 animals was calculated (Epitools[3]) to allow detection of an estimated prevalence of 25% with 5% precision with 95% confidence intervals. This would provide approximately 70 cases; providing 80% power to detect odds ratios of 2.5 or greater for exposures of 25% or greater in the control group.

## *7.3.2 Data collection*

Owners were instructed to house the animals and feed only a single slice of hay (approximately 1.5 Kg) on the evening before a visit.

### 7.3.2.1 Clinical examination and oral glucose test

Starting between 8am and 9am, each animal underwent a brief clinical examination to establish inclusion criteria were met and to estimate bodyweight (kg), calculated from girth circumference and body length (Carroll and Huntington, 1988). A blood sample (t=0) was collected into plain (Sarstedt[4]) and EDTA (Vacutainer[5]) evacuated tubes for measurement of serum insulin ($insulin_0$) and plasma ACTH concentrations respectively, and for patient-side measurement of blood glucose concentration ($glucose_0$). A feed containing 1 g/kg bodyweight glucose powder (Henry Schein[6]), mixed with 1 g/kg bodyweight low non-structural carbohydrate chaff (Happy Hoof[7]) and 1ml/kg bodyweight water was given. Time to consume the feed and the weight of any residual feed after 30 minutes were recorded. Following this, a physical examination was performed by a single, experienced veterinarian

blinded to questionnaire responses, which included BCS out of 9 (Kohnke, 1992), cresty neck score (Carter et al., 2009a), and semi-quantitative grades of supraorbital fat and hoof growth-ring morphology from 1 to 3 (Appendix 4). A second blood sample was collected for measurement of serum insulin concentration ($insulin_{120}$) and patient-side blood glucose concentration ($glucose_{120}$) 120 minutes after the feed. The animal was then returned to its normal management.

#### 7.3.2.2 Blood analysis

Following blood sampling EDTA tubes were placed immediately in crushed ice and serum tubes were allowed to clot at ambient temperature. Two to four hours following blood collection, tubes were  fuged at 3000 *g* for 10 min in a laboratory. Serum insulin concentration and, in animals aged ≥ 10 years, plasma ACTH concentration were measured in duplicate using chemiluminescent assays (Immulite 2000[8]/Immulite 2000XPi[8]) previously validated in horses (Perkins et al., 2002). Samples with an insulin concentration greater than the upper reportable limit (300µIU/mL) were diluted with charcoal-stripped serum (Chapter 3). The model of the chemiluminescent analyser was updated from the Immulite 2000 to the Immulite 2000XPi after 234 animals. To adjust for the new analyser paired measurement of insulin concentration in 39 samples was performed. Regression analysis (Appendix 5) was used to transform results from the Immulite 2000 to the newer Immulite 2000XPi, which are presented here. Blood glucose concentration was measured using a validated point of care glucometer (AlphaTRAK[9]) (Hackett and McCue, 2010).

#### 7.3.2.3 Survey data

During the visit, the owner or keeper of each horse completed a questionnaire by face-to-face interview by a single experienced research assistant. A range of mostly closed-end style questions were asked examining signalment, use, exercise duration and intensity, feeding and other management practices, and medical history (Appendix 6).

#### 7.3.2.4 Follow up

Following the visit, a report was sent to the horse's owner and their regular veterinary practice. For any horse testing positive for EMS or PPID advice on further diagnosis and management was enclosed.

### *7.3.3 Data Analysis*

A causal web for EMS was created (Appendix 7) allowing explanatory variables to be divided into two groups: potentially causal risk factors (e.g. age, management, season of testing, Table 1), and factors considered clinical manifestations that may be a result of EMS (e.g. hoof

changes, $insulin_0$, history of laminitis, Table 2). Two outcomes were examined: a binary outcome of EMS diagnosis where an animal was classified as positive for EMS if $insulin_0$ >33 µIU/mL, or $insulin_{120}$ >131 µIU/mL for animals consuming 75-100% of feed, or >112 µIU/mL for animals consuming 50 – 75% of feed (Durham et al., 2019) (animals consuming <50% of feed excluded); and a continuous outcome of $insulin_{120}$ where animals consuming <75% of feed were excluded (de Laat and Sillence, 2017). The continuous data were transformed using the natural logarithm of ($insulin_{120}$ + 1), to establish a normal distribution (Shaprio-Wilk). Animals were designated positive for PPID and thus excluded when plasma ACTH concentration was >50 pg/mL mid-November to mid-July and >100 pg/mL mid-July to mid-November (Schott et al., 2019). An ordinal hoof growth ring score (1-3) was calculated as the integer of the mean of the growth ring divergence and growth ring prominence scores. The hours exercised per week walking, trotting, cantering, jumping or galloping were multiplied by 1, 2, 4, 5 or 6, respectively, and the sum calculated as an overall exercise score. Sex was categorised as male and female due to low numbers of pregnant females and stallions (Table 1).

Due to the nature of sampling, data were clustered within horse yards (level two units) therefore multilevel (horse, yard) univariable logistic and linear regression models were used to identify explanatory factors associated with a binary outcome of EMS status (positive or negative) and a continuous outcome of $Log_e$ ($insulin_{120}$ + 1), respectively. Within-yard clustering was accounted for by incorporation of yard as a random intercept terms in all models. (Rasbach et al., 2017)

The prevalence of EMS was estimated after adjusting for clustering of animals within yard using the constant parameter estimate ($\beta_0$) and the standard errors derived from an intercept-only multilevel model incorporating yard as a random effect.

Potential correlations between explanatory variables were assessed using Pearson or Spearman coefficient according to the type and distribution of data. Correlated variables (correlation coefficient >0.7) were excluded or modified. Variables with a *P*-value <0.25 were entered into multilevel, multivariable models (Hosmer and Lemeshow, 2005). Variables with the greatest *P*-value were sequentially removed in a step-wise backwards elimination procedure with concurrent assessment for confounding, until all variables in the final model had *P*-values <0.05. Eliminated variables were individually inserted back into the

multivariable model to check if they improved the fit of the final model. Effect modifications were tested for clinically plausible interaction terms such as age*main activity and visit season*hours/day pasture in summer.

Associations between clinical manifestation variables and binary (EMS diagnosis) and continuous outcome ($Log_e$ ($insulin_{120}$ + 1)) were assessed using multilevel (horse, yard) univariable logistic and linear regression, respectively.

Data were analysed using commercial software MLwiN (version 3.01)[10] and SPSS (version 24)[11], and significance (2-sided) was assumed at $P$ <0.05.

### *7.3.4 Dates and location of the study and the candidate's role*

The study was performed in northwest England and north Wales from 2015-2016. The candidate contributed to study design and organised and performed data collection and sample analysis with assistance from technicians. He performed data analysis and prepared this chapter of the thesis.

## 7.4 Results

### *7.4.1 Study Population*

A total of 354 animals were examined at 64 properties. Of the establishments invited to participate, 19/151 studs, 19/154 livery yards and 26/61 riding schools were enrolled in the study, resulting in an overall establishment response rate of 17.5%. Median (range) animals included per establishment was 5 (1-12). Visits were conducted year-round from May 2015 to November 2016. Animals from which a second blood sample could not be obtained (n=2), and any positive for PPID (n=4) were excluded. Nine (2.6%) animals consumed <50% of feed and 19 animals (5.5%) consumed 50-75% of feed, resulting in sample sizes of n=339 for the binary outcome (EMS positive/negative) and n=320 for the continuous outcome ($log_e$ ($insulin_{120}$ + 1)).

In the binary outcome data set (n=339) median (IQR) age was 9 (6-11) years and 51.6% were geldings, 5.3% were stallions and 43.1% were mares, of which 9.6% were pregnant. Pure- or

crossbred Welsh (44.8%), Connemara (13.3%), Fell (3.8%), Shetland (3.8%), New Forest (2.9%), Highland (2.1%), Dartmoor (1.8%), Dales (1.5%) and Exmoor (0.9%) ponies, and cobs (25.1%) were included. Most animals (75.2%) were overweight (BCS 7-9), with the remainder (24.8%) ideal weight (BCS 4-6). Further descriptive data and univariable analysis are presented in Tables 1 and 2.

**Table 7.1**: Descriptive data for potentially causative risk factors for EMS in all (n=339), EMS positive (n=83) and EMS negative (n=256) ponies, univariable, multilevel logistic regression of potentially causal risk factors associated with a diagnosis of EMS (n=339) and univariable, multilevel linear regression of potentially causal risk factors associated with $Log_e$ ($insulin_{120}$ + 1, n=320) in native ponies and cobs in northwest England and north Wales. Categorical variables are presented as percentages, continuous variables as median (IQR)

| Variable | Category | All ponies (n=339) | Lower 95% CI | Upper 95% CI | EMS positive (n=83) | Lower 95% CI | Upper 95% CI | EMS negative (n=256) | Lower 95% CI | Upper 95% CI | Binary outcome Odds Ratio | Lower 95% CI | Upper 95% CI | *P*-Value | Continuous outcome Coefficient | S.E. | *P*-Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age (years)** | n/a | 9 (6 – 11) | | | 11 (9-13) | | | 8 (6 – 10) | | | 1.29 | 1.17 | 1.42 | <0.001 | 0.12 | 0.02 | <0.001 |
| **Sex (4 cat.)** | Gelding | 51.6 | 46.3 | 56.9 | 36.1 | 26.6 | 46.9 | 56.6 | 50.5 | 62.6 | Ref | | | 0.02 | Ref | | 0.006 |
| | Pregnant Mare | 4.1 | 2.5 | 6.8 | 6.0 | 2.6 | 13.3 | 3.5 | 1.9 | 6.6 | 2.89 | 0.75 | 11.2 | | 0.30 | 0.42 | |
| | Non-pregnant mare | 38.9 | 33.9 | 44.2 | 50.6 | 40.1 | 61.1 | 35.2 | 29.6 | 41.2 | 2.48 | 1.37 | 4.50 | | 0.54 | 0.16 | |
| | Stallion | 5.3 | 3.4 | 8.2 | 7.2 | 3.4 | 14.9 | 4.7 | 2.7 | 8.0 | 2.0 | 0.59 | 6.79 | | 0.54 | 0.38 | |
| **Sex** | Male | 56.9 | 51.7 | 62.2 | 43.4 | 32.7 | 54.0 | 61.3 | 55.4 | 67.3 | Ref | | | 0.003 | Ref | | 0.002 |
| | Female | 43.1 | 37.8 | 48.3 | 56.6 | 46.0 | 67.3 | 38.7 | 32.7 | 44.6 | 2.31 | 1.32 | 4.04 | | 0.47 | 0.15 | |
| **Pregnant** | No | 95.9 | 93.8 | 98.0 | 94.0 | 91.6 | 96.6 | 96.5 | 94.2 | 98.7 | Ref | | | 0.4 | Ref | | 0.9 |
| | Yes | 4.1 | 2.0 | 6.2 | 6.0 | 3.4 | 8.4 | 3.5 | 1.3 | 5.8 | 1.71 | 0.46 | 6.26 | | -0.05 | 0.42 | |
| **Breed** | Welsh A | 21.5 | 17.2 | 25.9 | 43.4 | 32.7 | 54.0 | 14.5 | 10.1 | 18.8 | Ref | | | <0.001 | Ref | | <0.001 |
| | Welsh B and C | 12.1 | 8.6 | 15.6 | 12.0 | 5.0 | 19.1 | 12.1 | 8.1 | 16.1 | 0.34 | 0.14 | 0.81 | | -0.75 | 0.24 | |
| | Welsh D | 11.2 | 7.9 | 14.6 | 9.6 | 3.3 | 16.0 | 11.7 | 7.8 | 15.7 | 0.27 | 0.11 | 0.69 | | -0.86 | 0.25 | |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cob | 25.1 | 20.5 | 29.7 | 8.4 | 2.5 | 14.4 | 30.5 | 24.8 | 36.1 | 0.09 | 0.04 | 0.24 | | -1.80 | 0.20 | |
| | Connemara | 13.3 | 9.7 | 16.9 | 4.8 | 0.2 | 9.4 | 16.0 | 11.5 | 20.5 | 0.1 | 0.03 | 0.32 | | -1.49 | 0.26 | |
| | Others[1] | 16.8 | 12.8 | 20.8 | 21.7 | 12.8 | 30.6 | 15.2 | 10.8 | 19.6 | 0.48 | 0.23 | 1.02 | | -0.73 | 0.26 | |
| **Visit season binary** | Dec - May | 31.9 | 26.9 | 36.8 | 31.3 | 21.3 | 41.3 | 32.0 | 26.3 | 37.7 | Ref | | | 0.8 | Ref | | 0.4 |
| | June-November | 68.1 | 63.2 | 73.1 | 68.7 | 58.7 | 78.7 | 68.0 | 62.3 | 73.7 | 1.11 | 0.55 | 2.27 | | 0.21 | 0.24 | |
| **Visit Season** | Mar – May | 15.9 | 12.4 | 20.2 | 16.9 | 10.3 | 26.3 | 15.6 | 11.7 | 20.6 | Ref | | | 0.8 | | | 0.5 |
| | Jun – Aug | 33.9 | 29.1 | 39.1 | 38.6 | 28.8 | 49.3 | 32.4 | 27.0 | 38.4 | 0.88 | 0.33 | 2.33 | | 0.41 | 0.34 | |
| | Sep – Nov | 34.2 | 29.4 | 39.4 | 30.1 | 21.3 | 40.7 | 35.5 | 30.0 | 41.6 | 0.73 | 0.33 | 1.62 | | 0.10 | 0.35 | |
| | Dec - Feb | 15.9 | 12.4 | 20.2 | 14.5 | 8.5 | 23.6 | 16.4 | 12.4 | 21.4 | 0.68 | 0.25 | 1.84 | | 0.12 | 0.40 | |
| **Est. Type** | Stud | 23.9 | 19.4 | 28.4 | 32.5 | 22.5 | 42.6 | 21.1 | 16.1 | 26.1 | Ref | | | 0.3 | Ref | | 0.07 |
| | Livery Yard | 23.0 | 18.5 | 27.5 | 21.7 | 12.8 | 30.6 | 23.4 | 18.2 | 28.6 | 0.61 | 0.25 | 1.46 | | -0.23 | 0.29 | |
| | Riding School | 53.1 | 47.8 | 58.4 | 45.8 | 35.1 | 56.5 | 55.5 | 49.4 | 61.6 | 0.55 | 0.26 | 1.18 | | -0.58 | 0.26 | |
| **Time to consume meal (min)** | n/a | 11 (8 – 15) | | | 11 (8-17) | | | 11 (8-14)) | | | 1.0 | 0.97 | 1.03 | 0.9 | 0.004 | 0.01 | 0.7 |
| **Percentage of meal consumed** | n/a | 100 (100-100) | | | 100 (100-100) | | | 100 (100 – 100) | | | 1.0 | 0.98 | 1.04 | 0.6 | -0.02 | 0.02 | 0.4 |
| **Main activity** | Show, breed, companion, pet | 29.5 | 24.6 | 34.4 | 43.4 | 32.7 | 54.0 | 25.0 | 19.7 | 30.3 | Ref | | | 0.005 | Ref | | <0.001 |
| | Riding or driving | 70.5 | 65.6 | 75.4 | 56.6 | 46.0 | 67.3 | 75.0 | 69.7 | 80.3 | 0.41 | 0.22 | 0.77 | | -0.77 | 0.19 | |
| **Exercise score[2]** | n/a | 11.4 (1.5 – 18.9) | | | 7.6 (0 – 15.6) | | | 13.0 (2.3 – 20) | | | 0.97 | 0.94 | 1.0 | 0.03 | -0.04 | 0.01 | <0.001 |
| **Weight status** | Under/normal (BCS 1-6) | 24.8 | 20.2 | 29.4 | 15.7 | 7.8 | 23.5 | 27.7 | 22.3 | 33.2 | Ref | | | 0.03 | Ref | | 0.004 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Overweight (BCS 7-9) | 75.2 | 70.6 | 79.8 | 84.3 | 76.5 | 92.2 | 72.3 | 66.8 | 77.7 | 2.20 | 1.07 | 4.50 | | 0.47 | 0.16 | |
| **Cresty Neck Score** | 1 and 2 | 34.8 | 29.7 | 39.9 | 20.5 | 11.8 | 29.2 | 39.5 | 33.5 | 45.4 | Ref | | | <0.001 | Ref | | <0.001 |
| | 3 | 40.1 | 34.9 | 45.3 | 30.1 | 20.3 | 40.0 | 43.4 | 37.3 | 49.4 | 1.27 | 0.62 | 2.59 | | 0.19 | 0.16 | |
| | 4 and 5 | 25.1 | 20.5 | 29.7 | 49.4 | 38.6 | 60.2 | 17.2 | 12.6 | 21.8 | 5.45 | 2.68 | 11.09 | | 0.82 | 0.18 | |
| **Hours/day at pasture summer** | 0-6 | 19.5 | 15.3 | 23.7 | 30.1 | 20.3 | 40.0 | 16 | 11.5 | 20.5 | Ref | | | 0.02 | Ref | | <0.001 |
| | 7-12 | 22.4 | 18.0 | 26.9 | 20.5 | 11.8 | 29.2 | 23 | 17.9 | 28.2 | 0.40 | 0.16 | 0.99 | | -0.52 | 0.28 | |
| | 12-24 | 58.1 | 52.9 | 63.4 | 49.4 | 38.6 | 60.2 | 60.9 | 55.0 | 66.9 | 0.35 | 0.17 | 0.74 | | -0.95 | 0.23 | |
| **Hours/day at pasture winter** | 0 | 15.0 | 11.2 | 18.8 | 18.1 | 9.8 | 26.4 | 14.1 | 9.8 | 18.3 | Ref | | | 0.4 | Ref | | 0.8 |
| | 1-6 | 30.1 | 25.2 | 35.0 | 33.7 | 23.6 | 43.9 | 28.9 | 23.4 | 34.5 | 1.05 | 0.42 | 2.64 | | -0.18 | 0.31 | |
| | 7-18 | 26.8 | 22.1 | 31.6 | 18.1 | 9.8 | 26.4 | 29.7 | 24.1 | 35.3 | 0.50 | 0.18 | 1.38 | | -0.22 | 0.33 | |
| | 19-24 | 28.0 | 23.2 | 32.8 | 30.1 | 20.3 | 40.0 | 27.3 | 21.9 | 32.8 | 0.94 | 0.38 | 2.37 | | -0.28 | 0.31 | |
| **Forage currently fed** | None | 5.9 | 3.4 | 8.4 | 2.4 | 0 | 5.7 | 7.0 | 3.9 | 10.2 | 0.31 | 0.05 | 1.77 | 0.02 | -0.46 | 0.39 | 0.03 |
| | Hay | 38.6 | 33.5 | 43.8 | 37.3 | 26.9 | 47.8 | 39.2 | 33.1 | 45.0 | Ref | | | | Ref | | |
| | Haylage | 51.6 | 46.3 | 56.9 | 50.6 | 39.8 | 61.4 | 52.0 | 45.8 | 58.1 | 0.98 | 0.50 | 1.90 | | -0.02 | 0.20 | |
| | Soaked Hay | 3.8 | 1.8 | 5.9 | 9.6 | 3.3 | 16.0 | 2.0 | 0.3 | 3.6 | 5.88 | 1.63 | 21.14 | | 0.98 | 0.37 | |
| **Forage weighed (n=319)** | No | 90.6 | 87.4 | 93.8 | 90.1 | 84.0 | 96.7 | 90.8 | 87.1 | 94.2 | Ref | | | 1.0 | Ref | | 0.6 |
| | Yes | 9.4 | 6.2 | 12.6 | 9.9 | 3.3 | 16.0 | 9.2 | 5.8 | 12.9 | 0.99 | 0.34 | 2.86 | | 0.21 | 0.36 | |
| **Number of additional feeds** | n/a | 1 (0-2) | | | 1 (0-1) | | | 1 (0-2) | | | 0.82 | 0.60 | 1.12 | 0.2 | -0.14 | 0.10 | 0.1 |
| | No | 70.5 | 65.6 | 75.4 | 68.7 | 58.7 | 78.7 | 71.1 | 65.5 | 76.6 | Ref | | | 0.9 | Ref | | |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Rug used in Summer** | Yes | 29.5 | 24.6 | 34.4 | 31.3 | 21.3 | 41.3 | 28.9 | 23.4 | 34.5 | 1.06 | 0.56 | 2.01 | | 0.14 | 0.19 | 0.4 |
| **Rug used winter** | No | 27.4 | 22.7 | 32.2 | 33.7 | 23.6 | 43.9 | 25.4 | 20.1 | 30.7 | Ref | | | 0.2 | Ref | | 0.02 |
| | Yes | 72.6 | 67.8 | 77.3 | 66.3 | 56.1 | 76.4 | 74.6 | 69.3 | 79.9 | 0.65 | 0.35 | 1.24 | | -0.47 | 0.19 | |

[1] Others: Fell (3.8%), Shetland (3.8%), New Forest (2.9%), Highland (2.1%), Dartmoor (1.8%), Dales (1.5%), and Exmoor (0.9%).

[2] Hours exercised per week walking, trotting, cantering, jumping or galloping were multiplied by 1, 2, 4, 5 or 6, respectively, and the sum calculated as overall exercise score.

**Table 7.2:** Descriptive data for clinical manifestations of EMS in all (n=339), EMS positive (n=83) and EMS negative (n=256) ponies; univariable, multilevel logistic regression of clinical manifestations associated with EMS diagnosis (n=339) and univariable, multilevel linear regression of clinical manifestations associated with $Log_e$ ($insulin_{120}$ + 1) (n=320) in native ponies and cobs in northwest England and north Wales. Categorical variables are presented as percentages with upper and lower 95% CI and continuous variables as median (IQR)

| Variable | Category | All ponies (n=339) (%) | Lower 95% CI | Upper 95% CI | EMS positive (n=83) (%) | Lower 95% CI | Upper 95% CI | EMS negative (n=256) (%) | Lower 95% CI | Upper 95% CI | Binary outcome Odds Ratio (95% CI) | Lower 95% CI | Upper 95% CI | *P*-Value | Continuous outcome Coefficient (95% CI) | S.E. | *P*-Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Laminitis in the last 5 years** | No | 90.3 | 87.1 | 93.4 | 69.9 | 60.0 | 79.7 | 96.9 | 94.7 | 99.0 | Ref | | | <0.001 | Ref | | <0.001 |
| | Yes | 9.7 | 6.6 | 12.9 | 30.1 | 20.3 | 40.0 | 3.1 | 1.0 | 5.3 | 14.40 | 5.87 | 35.28 | | 1.68 | 0.23 | |
| **Growth ring score** | 1 | 14.5 | 10.7 | 18.2 | 7.2 | 1.7 | 12.8 | 16.8 | 12.2 | 21.4 | Ref | | | 0.001 | Ref | | 0.009 |
| | 2 | 44.2 | 39.0 | 49.5 | 33.7 | 23.6 | 43.9 | 47.7 | 41.5 | 53.8 | 1.78 | 0.64 | 4.99 | | 0.38 | 0.21 | |
| | 3 | 41.3 | 36.1 | 46.5 | 59.0 | 48.5 | 69.6 | 35.5 | 29.7 | 41.4 | 4.80 | 1.62 | 12.40 | | 0.67 | 0.23 | |
| **Hoof pastern axis** | Straight | 76.1 | 71.6 | 80.6 | 71.1 | 61.3 | 80.8 | 77.7 | 72.6 | 82.8 | Ref | | | 0.2 | Ref | | 0.5 |
| | Broken Forward | 13.3 | 9.7 | 16.9 | 13.3 | 6.0 | 20.5 | 13.3 | 9.1 | 17.4 | 1.26 | 0.57 | 2.80 | | 0.087 | 0.21 | |
| | Broken Back | 10.6 | 7.3 | 13.9 | 15.7 | 7.8 | 23.5 | 9.0 | 5.5 | 12.5 | 2.04 | 0.92 | 4.53 | | 0.26 | 0.23 | |
| **Supraorbital fat score** | 1 | 18.3 | 14.2 | 22.4 | 13.3 | 6.0 | 20.5 | 19.9 | 15.0 | 24.8 | Ref | | | 0.004 | Ref | | 0.01 |
| | 2 | 49.0 | 43.6 | 54.3 | 37.3 | 26.9 | 47.8 | 52.7 | 46.6 | 58.9 | 1.01 | 0.48 | 2.15 | | 0.19 | 0.19 | |
| | 3 | 32.7 | 27.7 | 37.7 | 49.4 | 38.6 | 60.2 | 27.3 | 21.9 | 32.8 | 2.45 | 1.15 | 5.26 | | 0.58 | 0.21 | |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Blood glucose (t=0) (mmol/L)** | n/a | 5.0 (4.6 – 5.4) | | | 5.1 (4.6 – 5.4) | | | 5.0 (4.6 – 5.4) | | | 1.11 | 0.72 | 1.73 | 0.6 | 0.053 | 0.12 | 0.7 |
| **Change in Blood glucose (mmol/L)** | n/a | 3.0 (1.6 – 5.0) | | | 4.6 (3.2 – 6.0) | | | 2.5 (1.3 – 4.2) | | | 1.51 | 1.29 | 1.75 | <0.001 | 0.27 | 0.03 | <0.001 |
| **Insulin (t = 0 min) (µIU/mL)** | n/a | 0.0 (0.0 – 3.8) | | | 6.4 (2.0 – 15.4) | | | 0.0 (0.0 – 1.3) | | | | | | | | | |
| **$Log_e$ ($Insulin_0$ +1)** | n/a | 0.0 (0.0 – 1.6) | | | 2.0 (1.1 – 2.8) | | | 0.0 (0.0 – 0.8) | | | | | | | 0.048 | 0.006 | <0.001 |
| **Insulin (t=120 min) (µIU/mL)** | n/a | 45.7 (18.7 – 124.0) | | | 292.2 (180.8 – 477.0) | | | 30.5 (15.3 – 55.8) | | | | | | | | | |
| **$Log_e$ ($Insulin_{120}$ +1)** | n/a | 3.9 (3.0 – 4.8) | | | 5.7 (5.2 – 6.2) | | | 3.4 (2.8 – 4.0) | | | | | | | | | |

### *7.4.2 Binary outcome (EMS diagnosis)*

Eighty-three animals out of 339 were classified as positive for EMS. The overall prevalence of EMS adjusted for clustering within yard was 23.3% (95%CI 17.9–29.8%). In 9/339 (2.7%) animals $insulin_0$ was >33 µIU/mL; eight of these were also categorised as EMS positive based on $insulin_{120}$. Median (IQR) $insulin_{120}$ was 292 (181–477) µIU/mL in EMS positive animals and 30.5 (15.3–55.8) µIU/mL in EMS negative animals (Table 2).

#### 7.4.2.1 Risk factors

Univariable, multilevel logistic regression for risk factors (Table 1) resulted in 10 explanatory variables being entered a multivariable model, of which six were retained in the final model (Table 3). No clinically plausible, significant interaction terms were identified. The odds of a positive diagnosis of EMS increased by 1.38 (95% CI 1.24–1.54) with each year increase in a pony's age. Female animals and those involved in more sedentary main activities including showing, breeding and being companions had increased odds of being positive for EMS compared to those ridden or driven. Compared to the Welsh section A, the other Welsh, Connemara and cob breeds all had decreased odds of EMS. Furthermore, animals with a BCS ≥7/9 and those that were turned out to pasture for shorter periods during the summer had increased odds of being positive for EMS.

#### 7.4.2.2 Clinical manifestations

Univariable, multilevel logistic regression (Table 2) showed that animals reported to have had at least 1 episode of laminitis during the last 5 years were 14.4 (95% CI 5.9–35.3) times more likely to be positive for EMS than those with no recent history of laminitis. Growth ring and supraorbital fat scores of 3/3 were both associated with increased odds of EMS compared to scores of 1/3. A greater increase in blood glucose concentration ($glucose_{120}$–$glucose_0$) was associated with increased odds of EMS, however $glucose_0$ was not.

**Table 7.3:** Multivariable, multilevel logistic (n=339) and linear (n=320) regression models of risk factors associated with EMS diagnosis and $Log_e$ ($insulin_{120}$ + 1), respectively, in native ponies and cobs in northwest England and north Wales.

| Variable | Category | Logistic regression (n=339) | | | | Linear regression (n=320) | | |
|---|---|---|---|---|---|---|---|---|
| | | OR | Lower 95% CI | Upper 95% CI | *P* -value | Coefficient | S.E. | *P*-value |
| **Age** | Years | 1.38 | 1.24 | 1.54 | <0.001 | 0.13 | 0.02 | <0.001 |
| **Sex** | Male | Ref | | | 0.02 | Ref | | 0.02 |
| | Female | 2.13 | 1.13 | 4.01 | | 0.27 | 0.12 | |
| **Breed** | Welsh A | Ref | | | <0.001 | Ref | | <0.001 |
| | Welsh B and C | 0.35 | 0.13 | 0.93 | | -0.60 | 0.21 | |
| | Welsh D | 0.15 | 0.05 | 0.45 | | -0.79 | 0.22 | |
| | Cob | 0.14 | 0.05 | 0.39 | | -1.35 | 0.18 | |
| | Connemara | 0.06 | 0.02 | 0.20 | | -1.48 | 0.22 | |
| | Others | 0.51 | 0.21 | 1.20 | | -0.58 | 0.19 | |
| **Main Activity** | Riding / driving | Ref | | | <0.001 | Ref | | <0.001 |
| | Showing/breeding/companion/other | 3.51 | 1.75 | 7.04 | | 0.66 | 0.16 | |
| **Time at grass in summer (hours)** | 0-6 | Ref | | | <0.001 | Ref | | <0.001 |
| | 7 – 12 | 0.25 | 0.10 | 0.64 | | -0.46 | 0.21 | |

| | >12 | 0.21 | 0.10 | 0.47 | | -0.91 | 0.17 | |
|---|---|---|---|---|---|---|---|---|
| **Overweight (BCS ≥7/9)** | No | Ref | | | 0.003 | Ref | | <0.001 |
| | Yes | 3.48 | 1.53 | 7.92 | | 0.48 | 0.13 | |
| **Winter Rugging** | No | | | | n/s | Ref | | 0.02 |
| | Yes | | | | | -0.36 | 0.16 | |
| **Within-yard variance (S.E.)** | | 0.000 (0.000) | | | | 0.139 (0.059) | | |

### *7.4.3 Continuous Outcome (Log$_e$ (insulin$_{120}$ + 1), n=320)*

Median (IQR) insulin$_{120}$ for all animals was 50.3 (19.9–127.8) µIU/mL (Fig. 1).

#### 7.4.3.1 Risk factors

Univariable, multilevel linear regression (Table 1) resulted in 10 explanatory variables being entered into a multivariable model. Seven factors were retained in the final model (Table 3). In addition to the six variables identified by binary logistic regression, winter rugging was associated with decreased Log$_e$ (insulin$_{120}$ + 1); with an estimated coefficient (s.e.) of -0.36 (0.16).

#### 7.4.3.2 Clinical manifestations

Univariable, multilevel linear regression is provided in Table 2. The same clinical manifestations were associated with increased Log$_e$ (insulin$_{120}$ + 1) as were associated with a positive EMS diagnosis.

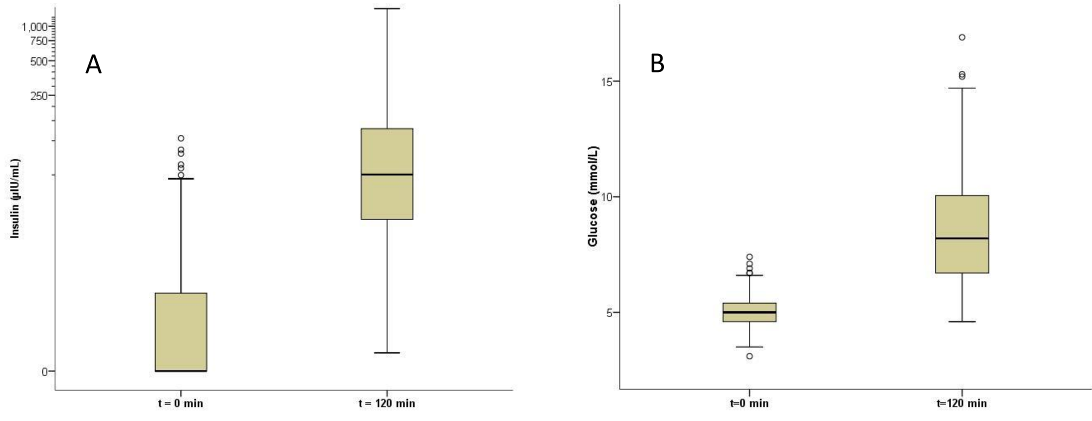



**Fig. 7.1:** Box and whisker plot of serum insulin (A) and blood glucose (B) concentrations after fasting (t = 0 min) and 120 minutes following 1 g/kg glucose (t = 120 min) in 320 ponies in northwest England and north Wales.

## 7.5 Discussion

This study represents the first robust prevalence estimate of EMS in susceptible UK breeds using a dynamic, oral test for ID. The prevalence of 23.3% demonstrates that a significant proportion of 3-14-year-old native ponies and cobs in the UK are at increased risk of developing laminitis. After allowing for fixed effects in the multivariable models there was minimal remaining within-yard clustering with estimates of zero for the binary model and 0.14 for the continuous model (Table 3), suggesting the fixed effects such as breed and main activity account for much of the yard-level variation. Studies in Australia on mixed breed ponies (Morgan et al., 2014) and in the USA on light breed horses (Pleasant et al., 2013) estimated the prevalence of EMS to be 27% and 18%, respectively, based on a basal insulin of >20 µIU/mL. Applying the same diagnostic criteria in the current study would have resulted in a much lower prevalence of EMS (5.7%), likely reflecting differences in the period of fasting and the insulin assay used, as well as between the populations studied.

Several diagnostic cut-offs for basal fasting and post-OGT insulin concentrations for EMS have been published (Carter et al., 2009c, Frank et al., 2010), and in a recent study, thresholds were calculated that quantified laminitis risk in ponies subsequently fed a high carbohydrate diet (Meier et al., 2018). In our study, the cut-off was taken from a recent consensus document (Durham et al., 2019) using an equivalent assay. Different cut-offs may have resulted in a different prevalence and possibly associated risk factors for a positive diagnosis but would not have affected the risk factors associated with $insulin_{120}$. Although EMS is defined as collection of historical, clinical and laboratory risk factors for endocrinopathic laminitis, ID is the central and consistent feature (Durham et al., 2019), and increasingly the terms ID and EMS are being used interchangeably. Because of this, in the current study a diagnostic test for ID was used to determine EMS status. In clinical practice a diagnosis of EMS is primarily used to identify horses at increased risk of potentially life-threatening laminitis. This, and the fact that management of EMS is centred on low-risk and inexpensive modification of diet and exercise means that the false negative rate should be minimised by using cut-offs which prioritise sensitivity over specificity. It is important to consider the insulin assay being used, as results can vary considerably (Chapter 3).

Equine metabolic syndrome increases risk for endocrinopathic laminitis, so the prevalence of EMS and laminitis are related but not directly comparable. In this study 9.7% of all ponies

were reported to have had at least 1 episode of laminitis in the previous 5 years, and of those, 76% were EMS positive. Veterinary advice was sought in 56% of the reported episodes of laminitis. The frequency of previous laminitis may be an underestimate, as the median (IQR) duration of ownership of all animals was 3 (1-6) years. An episode of laminitis during the previous 5 years was strongly associated with an increased risk of current EMS, indicating that animals are at greater risk of laminitis if they have had an episode in the last 5 years. Other estimates of the frequency of endocrinopathic laminitis in equids vary considerably, ranging from 1.5% to 34% (Wylie et al., 2011). In most studies laminitis is defined by foot lameness as detected by the owner. Divergent hoof growth rings have been shown previously to occur in feet with histological evidence of laminitis (Karikoski et al., 2015), and the association between hoof ring score and EMS and $insulin_{120}$ shown in this study further supports this. The use of hoof morphology as a simple method of identifying animals at risk of EMS is poorly described and requires further research.

Several risk factors associated with increased $insulin_{120}$ and a diagnosis of EMS support findings from previous studies. Increasing age was identified as a risk factor for EMS, supporting the association between age and ID that has been recognised in humans and horses (Morgan et al., 2014, Karakelides et al., 2010). This study also supports the association between adiposity and EMS, with both obesity (BCS ≥7/9) and CNS increasing the odds of EMS in the univariable analysis, and obesity remaining in both multivariable models. CNS was excluded from the multivariable model only because of a strong correlation with obesity. The causal relationship between obesity and EMS remains unclear, but a proinflammatory state (Vick et al., 2007) and dysregulation of adipokines, for example adiponectin and leptin (Pleasant et al., 2013, Menzies-Gow et al., 2017) are likely. An association between BCS and ID has been lacking in some smaller experimental studies (Fitzgerald et al., 2019a), while others have demonstrated an effect of diet (Bamford et al., 2016a) or regional adiposity (Carter et al., 2009c, Fitzgerald et al., 2019a). Although BCS is an imperfect predictor of total body fat (Dugdale et al., 2012) these results support its use as a modifiable risk factor and to screen for animals at increased risk of EMS.

This study chose breeds considered at increased risk of EMS, and in which previous UK-based research is lacking. These results may not be transferrable to other breed groups. This focused approach did allow the study to show variation in risk between the breeds studied, with an increased odds of EMS in the smaller Section A compared with the other sections

within the Welsh mountain pony breed. Other studies have found an inverse correlation between height and baseline insulin, driven, in part by genetic factors (Norton et al., 2019a). Some breed groups including Highland ponies and Shetlands were less well represented, potentially limiting the power to detect breed differences in these breeds.

This is the first study to show a sex effect with mares having greater odds of EMS than geldings and stallions. Insulin antagonism by female sex hormones might contribute to this finding (Godsland, 2005). Although pregnancy has been cited as contributing to ID in people (Caughey and Turrentine, 2018) this is less likely in horses, particularly during the first and second trimesters of pregnancy (Beythien et al., 2017). Pregnancy was not found to increase the odds of EMS in the current study although only 14 (4.1%) of the animals were pregnant.

Ponies and cobs with sedentary main activities including showing, breeding and being companions had increased odds of being positive for EMS compared to those ridden or driven. Although the exercise score was significantly associated with EMS and $insulin_{120}$ in the univariable models, it was only the main activity that was retained in the final models. Previous research has been conflicting as to the level of exercise required to improve ID. Moderate intensity exercise has been recommended by consensus [1], but even very low levels of exercise have also been shown to affect morphological indicators of obesity (Bamford et al., 2019), which may indirectly decrease the risk of EMS. The results of this study support exercise as a modifiable factor than could be used to decrease the odds of EMS.

Absence of rugging in winter and decreased turnout at pasture in the summer were two novel risk factors associated with increased $insulin_{120}$ and risk of EMS identified in this study. These contradict most current opinion (Durham et al., 2019), although these data only estimate association, not causality and it is possible that they represent owners identifying animals at increased risk of EMS (due to obesity or previous laminitis) and modifying management according to current advice of keeping horses unrugged to promote cold-induced thermogenesis and restricting summer turn out to reduce grass intake. There was no significant association between previous laminitis and winter rugging or summer turnout at pasture identified on chi-squared analysis (data not shown). The rationale for leaving a horse unrugged is dependent on the ambient temperature being lower than the animal's lower critical temperature (LCT), meaning that additional energy is used to maintain core body temperature. Estimates of the horse's LCT vary between 0-5°C (Morgan, 1998), but horses

will adapt to their environment. Mean minimum daily air temperature during January across the region studied is typically 2°C (Met Office, 2016), close to the LCT. It is also possible that rugging a horse in cold weather might increase movement and hence exercise. Clipping the coat is probably more common in horses engaged in strenuous activities, and means they are more likely to be rugged. Although an exercise score and main activity were included in the analyses, clipping is a possible confounding factor in the observed association between absence of rugging and EMS. This warrants further research.

A limitation and source of potential bias in this study was the incomplete consumption of glucose by some animals. The OGT was selected for diagnosis of EMS as it is a dynamic test that assesses the enteroinsular axis as well as insulin resistance and is practical to use in the field. It has reasonable repeatability (de Laat and Sillence, 2017) and has been shown to predict laminitis risk in ponies fed a high carbohydrate diet (Meier et al., 2018). All animals consuming >75% of glucose were classified together for EMS diagnosis cut-off and included in the analysis for the outcome of $insulin_{120}$. A previous study (de Laat and Sillence, 2017) unexpectedly found that insulin responses were greater following 0.75 g/kg compared to 1 g/kg glucose dose. Intestinal absorption of glucose might be saturated at doses >0.75 g/kg, and the authors in the study above speculated that differences in consumption rate, or other gastrointestinal factors might have contributed. In the current study the exclusion of animals consuming <75% and <50% of the glucose from the $insulin_{120}$ and EMS diagnosis outcomes, respectively, might have introduced bias or imprecise prevalence estimates. Willingness to voluntarily consume an unfamiliar feed might be associated with obesity and ID, overestimating the prevalence of EMS. Additionally, owners of obese or previously laminitic animals might have been more likely to volunteer, introducing selection bias.

In conclusion, this study shows there is a high prevalence of EMS and increased risk of laminitis in native ponies and cobs aged 3-14 years in a specific region of the UK. Several historical, management and morphological risk factors are identified that owners and veterinarians can use to identify animals that are at increased risk of EMS, allowing more accurate selection of animals for management based on a presumptive diagnosis, or for further diagnostic testing. It also highlights modifiable risk factors to assist in the management of EMS. Further prospective studies are warranted to determine whether novel associated risk factors such as decreased summer grazing are causal, or a result of reactive management changes by owners.

# 7.6 Manufacturers' addresses

[1] BHS, Kenilworth, UK

[2] https://www.wirralhorse.co.uk/

[3] Epitools Epidemiological Calculators, Ausvet, Australia.

[4] Sarstedt, Leicester, UK

[5] Becton-Dickinson, Oxford, UK

[6] Henry Schein UK Holdings, Gillingham, UK

[7] Spillers, Milton Keynes, UK

[8] Siemens, Healthcare, Camberley, UK

[9] Zoetis, London, UK

[10] Centre for Multilevel Modelling, University of Bristol, UK

[11] IBM Corp., New York

# Chapter 8: General discussion and concluding comments

## 8.1 Overview

The studies presented in this thesis sought to enable improvements in the diagnosis of Equine Metabolic Syndrome, thus enabling more targeted and earlier implementation of management strategies to help prevent laminitis. Additionally, studies identifying modifiable risk factors for EMS and examining the insulinaemic response to different feeds aimed to help guide management of EMS-positive horses.

## 8.2 Sampling handling and insulin assay validation

Laboratory testing for EMS centres on the assessment of insulin dysregulation, usually involving measurement of serum or plasma insulin concentration. Chapter 2 assessed the effect of different storage and handling conditions commonly available in first opinion clinical practice and showed that neither separation of serum nor storage at room temperature had a significant effect. This is a reflection not only of the stable immunoreactivity of the insulin molecule, but also of the insignificant accumulation of interfering peptides, and these results have been supported by another study published since (Leschke et al., 2019). It is rare for an insulin analyser to be available close to the site of sampling in clinical practice, and these results will enable simpler and cheaper handling of blood samples in both clinical practice and field research.

In Chapter 3, the Immulite 2000 chemiluminescent insulin assay (CLIA), used in many commercial diagnostic laboratories in the UK was validated, and showed strong correlation and adequate agreement with the Coat-a-count radioimmunoassay (RIA) at the most important diagnostic concentrations (<100µIU/mL). Several landmark studies in the period between 2000 and 2010 (Carter et al., 2009c, Treiber et al., 2005, Treiber et al., 2006) used the validated (Tinworth et al., 2011) Coat-a-count RIA for measurement of insulin concentration, and many of the diagnostic criteria used in clinical practice have been derived from this work. Difficulties with handling radioisotopes and with processing frequent, small numbers of samples mean that RIA is rarely used in diagnostic laboratories, added to which the Coat-a Count was discontinued in the early 2010s. By contrast, the Immulite 2000 CLIA was favoured by diagnostic laboratories as it offered rapid, highly automated analysis, without the difficulties associated with handling radioisotopes and batch sampling. The good intra- and interassay precision of the Immulite analyser demonstrated in this study means

that insulin concentrations in an individual can be reliability monitored over time, for example in response to management modification. If dilution of serum samples is required to bring insulin concentration within the detectable limits of the assay, then charcoal stripped serum should be used as the diluent, and the lowest dilution factor possible should be used. When applying diagnostic criteria derived on the Coat-a-Count RIA to results from the Immulite 2000 CLIA, at concentrations <100µIU/mL results from the CLIA are slightly less than from the RIA, and as a result the Immulite 2000 has a slightly lower sensitivity for detecting low concentrations of insulin. This might limit its diagnostic utility when dichotomising results using very low diagnostic thresholds, for example 5.3µIU/mL for fasting insulin (Olley et al., 2019).

Part-way through this thesis the model of the CLIA analyser was updated, and the Immulite2000 was replaced with the Immulite2000xpi. Despite the same antibodies and reagent kits being used, and excellent agreement for the human standards provided, locally stored equine standards at a wide range of concentrations all showed an increase in insulin concentrations when measured on the Immulite 2000xpi. A comparison between the analysers in Chapter 7 showed a very strong correlation, and in that chapter results from the Immulite 2000 were transformed to Immulite2000xpi results for analysis. This difference between the two versions of the same analyser highlights the importance of using equine-specific standards for assessment of precision.

A major obstacle when evaluating equine insulin assays is the lack of an equine insulin standard solution, a reference standard insulin assay other than liquid chromatography-mass-spectrometry (LC-MS), or an immunoassay with antibodies raised specifically against equine insulin. One ELISA has been optimised for equine insulin (Öberg et al., 2011) but uses antibodies generated against porcine insulin and porcine insulin standards. Although there is no universal standardisation of human insulin assays (Staten et al., 2010) there is a World Health Organisation International standard for human insulin (Moore et al., 2019a). Smaller studies comparing assays for measurement of equine insulin will continue to provide useful data, but until a full standardisation of insulin assays using an equine insulin standard of known concentration is performed, inaccuracies will continue when translating results between studies and assays.

Data from Chapters 2 and 3 was used to inform sample handling and analysis in chapters 4-7.

## 8.3 Comparison of tests for insulin dysregulation

The studies presented in Chapters 4-6 were based on a seven-way, randomised cross-over study performed in 12 ponies. Chapter 4 addressed the objective of comparing diagnostic tests for insulin dysregulation in ponies, including one novel test, using a proprietary breakfast cereal (WEET). Tests intended for use in a clinical setting have several practical requirements. In this respect, the WEET test offered the advantage over the oral glucose test (OGT) and oral sugar test (OST) that the carbohydrate source quality assured by the manufacturer to human standards, easily purchased, measured (in number of biscuits) and prepared by horse owners. The major disadvantage of WEET compared to the other oral tests, however, was the poor palatability, and more extensive pilot studies may have allowed alteration of the presentation, or use of a lower dose or another, more palatable cereal product. Despite the variable consumption, all ponies in this study showed an insulinaemic response to the WEET which was strongly correlated to the insulinaemic response from other oral tests and was different between insulin resistant and sensitive groups. There is scope of further work developing a more palatable and easily obtained oral carbohydrate source for assessment of ID.

The expression of the different components (basal hyperinsulinaemia, postprandial hyperinsulinaemia and insulin resistance) of insulin dysregulation varies between affected individuals (Frank and Tadros, 2014). The diagnostic tests included in Chapter 4 assessed different components of ID: the CGIT assessed insulin resistance only, fasting insulin assessed basal hyperinsulinaemia and the oral dynamic tests assessed both postprandial hyperinsulinaemia, including the contribution of the enteroinsular axis (EIA), and insulin resistance. All ponies that were positive to the CGIT (n=4) were also positive to the OGT indicating that in these horses either insulin resistance was the major contributor to a positive OGT result, or that insulin resistance and over-expression of the EIA coexisted. These results suggest that performing both the CGIT and the OGT will have limited use for increasing the overall sensitivity for detection of ID. Fasting basal insulin showed a very low sensitivity for detection of ID compared to oral test and CGIT, and data from both Chapters 4 and 7 indicate there is minimal diagnostic advantage to be gained from measuring fasting basal as well as post-carbohydrate insulin concentration. When applied to clinical practice this has the advantage of reducing the veterinary time required to perform the test. Maximising the diagnostic accuracy of fasting insulin as a diagnostic test for ID necessitates use of a very low diagnostic threshold (Olley et al., 2019), close to the minimum detectable concentration for

many assays and therefore a potential source of inaccuracy. The clustering of fasting basal insulin concentrations at very low values (Chapter 7) increases the risk of discordant results between assays being introduced by fixed bias. In addition, insulin sensitivity is reduced by fasting (Bertin et al., 2016), potentially confounding results. Recently, measurement of basal insulin while the animal is consuming their normal forage diet has been recommended (Durham et al., 2019), as it acts as a low-glycaemic oral challenge and more accurately reflects the day-to-day insulinaemic status of the animal.

In the OGT-positive, CGT-negative horses (n=2), there was no difference seen in glucagon-like peptide-1 (GLP-1) response in Chapter 6 to explain the discrepancy in results. It is possible that the hyperinsulinaemia following oral glucose was a result of another part of the EIA, such as glucose-dependent insulinotropic peptide (GIP), or the study lacked power to show a difference. Alternatively, these two cases could reflect low specificity of the OGT, and they represent false positives.

The euglycaemic hyperinsulinaemic clamp is established as the reference or 'gold' standard test for the diagnosis of tissue insulin resistance (Bertin and de Laat, 2017), but there is no established, equivalent status diagnostic test for ID which incorporates the EIA.

## 8.4 Glycaemic and insulinaemic responses to preserved forages

Given the poor sensitivity of fasting insulin for diagnosis of ID, measurement of basal insulin concentration during grazing or consumption of preserved forage has been recommended when dynamic tests are not available (Durham et al., 2019, Frank et al., 2020). This recommendation is supported by results from Chapter 5 showing that insulin responses to dry hay, soaked hay and haylage were greater in ID compared to non-ID ponies, as defined by an OGT. Although meal fed, the marked difference in insulin response between the three forages highlights that if basal insulin concentration during consumption of forages is to be used to diagnose ponies as ID or non-ID, then thresholds for diagnosis should consider the NSC content and other factors in the of the forage such as fermentation products or digestibility. As an illustration, the median (IQR) maximum insulin concentration following soaked hay in ID positive animals was 15.5(13.2) µIU/mL, whereas following haylage in ID-negative animals it was 26(46) µIU/mL.

Reducing post-prandial insulinaemia is a major objective in the management of EMS-positive animals. The effect of soaking hay on reducing postprandial insulin response shown in Chapter 5 is supportive of this practice. The greater insulinaemic response following haylage compared to hay or soaked hay contradicted the hypotheses of the study. The batch of haylage used in the study had a higher NSC (18.5%) than expected based on the manufacturer's data and is likely to have been a major determinant of the insulin response. The measurement of the NSC in the forages in this study used wet chemistry, considered to be the reference standard for forages, but variation in wet chemistry results and variation of wet chemistry from more commonly used near infrared spectrometry has been reported (Harris et al., 2018). This highlights an area of concern as owners will typically use manufacturer data.

Although higher than the manufacturer's data, the wet chemistry measured NSC was similar to the wet chemistry measured unsoaked hay and there was a particularly marked insulin response prompting consideration of the effect of fermentation products. In a study published subsequently (Lindase et al., 2018) horses showed a much greater insulinaemic response after feeding a very low NSC haylage compared with the response to soaked hay seen in Chapter 5, despite very similar total NSC doses (0.25 g/kg BW and 0.27 g/.kg BW, respectively). Differences in breed and insulin assay affect comparison of these studies, but further work on the direct insulinaemic properties of grass fermentation products such as volatile fatty acids and ethanol, or further comparisons of the insulinaemic effects of fermented and dried forages is warranted.

## 8.5 Glucagon-Like peptide response to oral carbohydrates

To further investigate the role of the EIA in postprandial insulinaemia, Chapter 6 examined potential associations between GLP-1 response and the oral carbohydrate fed, ID status, and insulin response. Although the GLP-1 response to haylage was greater than to soaked hay, no difference between haylage and hay was detected, indicating that the marked insulinaemic response to haylage seen in Chapter 5 was unlikely to have been driven by GLP-1. There was no difference in GLP-1 responses between ID-positive and ID-negative groups, whether defined by OGT or CGIT, and the results of this study do not support the use of GLP-1 for the diagnosis of ID, based on current understanding and definitions. There was a strong

positive corelation between GLP-1 and insulin, however, both at individual time points and as AUC. Equine pancreatic islets *in vitro* increase insulin secretion in response to human GLP-1 (Kheder et al., 2018), and it is likely that the association between GLP-1 and insulin concentration identified in Chapter 6 resulted partly from the insulinotropic effects of GLP-1 (de Laat et al., 2016). A greater influence is likely to have been a simultaneous dose-dependent effect of oral carbohydrate on both GLP-1 concentration (Yoder et al., 2010) and blood glucose–dependent insulin secretion. GLP-1 is estimated to cause 23% of insulin secretion following oral carbohydrate in horses, (de Laat et al., 2016), but this is likely to be variable between individuals. The failure to detect an association between GLP-1 and ID status might have been caused by insufficient statistical power, variability in GLP-1 secretion or in its insulinotropic effects relative to enteric glucose absorption, or variation in other, unidentified influences on insulin secretion between individuals. In future, measurement of GLP-1 concentration might a have role alongside other tests to provide a holistic assessment of ID status. It is possible that GIP, the other major incretin, which was not included in Chapter 6, or other parts of the EIA have a greater role than previously identified (de Laat et al., 2016). Multiple extra-pancreatic effects of GLP-1 and GIP are described in humans, and further research is required to explore the contribution of these to postprandial insulin concentrations in horses.

## 8.6 Epidemiology of EMS

In Chapter 7, a cross sectional study was performed with the aim of estimating prevalence of EMS in UK native ponies and cobs aged 3-15 years and identifying associated risk factors. Experimental studies examining risk factors for EMS and smaller surveys of prevalence had been published, but there had been no large-scale epidemiological study using a dynamic test for EMS, and epidemiological data on EMS was lacking (Durham et al., 2019). The population was selected as there is anecdotal and published (Bamford et al., 2014, Norton et al., 2019b) evidence that pony breeds are at higher risk of EMS, and the age range as well as basal ACTH measurement meant there was a low chance of results being confounded by pituitary *pars intermedia* dysfunction. The results are applicable to the defined population, but prevalence and risk factors might be different in other breeds, ages and locations. The OGT was chosen for assessment of ID based on the comparison to the CGIT and other tests in Chapter 4, its inclusion of the EIA and acceptability to owners. Subsequently, similar tests

have been shown to be repeatable (de Laat and Sillence, 2017) and predictive of laminitis in ponies fed an experimental diet high in NSC (Meier et al., 2018).

Results confirmed that EMS is highly prevalent in this population of animals and strategies for prevention and management of the syndrome are warranted to improve equine welfare. Associated risk-factors identified included age, Welsh Section A breed and females. A more sedentary main activity and obesity were identified as modifiable risk-factors and will help inform preventative strategies and management. Novel risk factors related to rugging in winter and time on pasture in summer were identified, but further prospective work is required to determine if these were causal or a result of reactive management changes by owners. Clinical manifestation associated with EMS included divergence and prominence of the growth rings of the hoof, in the absence of detectable foot pain. These findings, alongside other studies demonstrating that histological evidence of laminitis precedes the onset of detectable pain (Karikoski et al., 2015), require further investigation, and potentially a reassessment of how laminitis is defined clinically.

The results of this study also mean that the probability of EMS in this group is moderately high and use of diagnostic screening tests and/or modification of management of at-risk ponies and cobs, particularly with the risk factors identified above, is warranted.

## 8.7 Conclusions

Equine metabolic syndrome is highly prevalent in UK ponies and cobs, and management and phenotypic features exist to identify animals at increased risk. Ideally, in addition to tests of insulin resistance, further diagnostic testing should include an assessment of the response to oral carbohydrate to ensure all positive animals are captured. Future studies should aim to identify the cause of postprandial hyperinsulinaemia in the sub-group of animals with apparently normal incretin responses and tissue insulin sensitivity. Modifiable risk factors exist to guide management of EMS, and dietary modification is indicated to help reduce hyperinsulinaemia.

# References


ABDUL-GHANI, M. A. & DEFRONZO, R. A. 2010. Pathogenesis of Insulin Resistance in Skeletal Muscle. *J Biomed and Biotech,* 2010**,** 476279.

ANDREAZZI, A. E., GRASSIOLLI, S., MARANGON, P. B., MARTINS, A. G., DE OLIVEIRA, J. C., TORREZAN, R., GRAVENA, C., GARCIA, R. M. & MATHIAS, P. C. 2011. Impaired sympathoadrenal axis function contributes to enhanced insulin secretion in prediabetic obese rats. *Exp Diabetes Res,* 2011**,** 947917.

ARGENZIO, R. A. & HINTZ, H. F. 1970. Glucose tolerance and effect of volatile fatty acid on plasma glucose concentration in ponies. *J Anim Sci,* 30**,** 514-8.

ARGO, C. M., CURTIS, G. C., GROVE-WHITE, D., DUGDALE, A. H., BARFOOT, C. F. & HARRIS, P. A. 2012. Weight loss resistance: a further consideration for the nutritional management of obese Equidae. *Vet J,* 194**,** 179-88.

ARGO, C. M., DUGDALE, A. H. & MCGOWAN, C. M. 2015. Considerations for the use of restricted, soaked grass hay diets to promote weight loss in the management of equine metabolic syndrome and obesity. *Vet J,* 206**,** 170-7.

ASPLIN, K. E., SILLENCE, M. N., POLLITT, C. C. & MCGOWAN, C. M. 2007. Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. *Vet J,* 174**,** 530-5.

BAGGIO, L. L. & DRUCKER, D. J. 2007. Biology of incretins: GLP-1 and GIP. *Gastroenterology,* 132**,** 2131-57.

BAILEY, S. R., HABERSHON-BUTCHER, J. L., RANSOM, K. J., ELLIOTT, J. & MENZIES-GOW, N. J. 2008. Hypertension and insulin resistance in a mixed-breed population of ponies predisposed to laminitis. *Am J Vet Res,* 69**,** 122-9.

BAMFORD, N. J., BASKERVILLE, C. L., HARRIS, P. A. & BAILEY, S. R. 2015. Postprandial glucose, insulin, and glucagon-like peptide-1 responses of different equine breeds adapted to meals containing micronized maize. *J Anim Sci,* 93**,** 3377-83.

BAMFORD, N. J., POTTER, S. J., BASKERVILLE, C. L., HARRIS, P. A. & BAILEY, S. R. 2016a. Effect of increased adiposity on insulin sensitivity and adipokine concentrations in different equine breeds adapted to cereal-rich or fat-rich meals. *Vet J,* 214**,** 14-20.

BAMFORD, N. J., POTTER, S. J., BASKERVILLE, C. L., HARRIS, P. A. & BAILEY, S. R. 2019. Influence of dietary restriction and low-intensity exercise on weight loss and insulin sensitivity in obese equids. *J Vet Intern Med,* 33**,** 280-286.

BAMFORD, N. J., POTTER, S. J., HARRIS, P. A. & BAILEY, S. R. 2014. Breed differences in insulin sensitivity and insulinemic responses to oral glucose in horses and ponies of moderate body condition score. *Domest Anim Endocrinol,* 47**,** 101-7.

BAMFORD, N. J., POTTER, S. J., HARRIS, P. A. & BAILEY, S. R. 2016b. 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. *Equine Vet J,* 48**,** 368-73.

BANSE, H. E., HOLBROOK, T. C., FRANK, N. & MCFARLANE, D. 2016. Relationship of skeletal muscle inflammation with obesity and obesity-associated hyperinsulinemia in horses. *Can J Vet Res,* 80**,** 217-24.

BANSE, H. E., MCCANN, J., YANG, F., WAGG, C. & MCFARLANE, D. 2014. Comparison of two methods for measurement of equine insulin. *J Vet Diagn Invest,* 26**,** 527-530.

BERTIN, F. R. & DE LAAT, M. A. 2017. The diagnosis of equine insulin dysregulation. *Equine Vet J,* 49**,** 570-576.

BERTIN, F. R. & SOJKA-KRITCHEVSKY, J. E. 2013. Comparison of a 2-step insulin-response test to conventional insulin-sensitivity testing in horses. *Domest Anim Endocrinol,* 44**,** 19-25.
BERTIN, F. R., TAYLOR, S. D., BIANCO, A. W. & SOJKA-KRITCHEVSKY, J. E. 2016. The Effect of Fasting Duration on Baseline Blood Glucose Concentration, Blood Insulin Concentration, Glucose/Insulin Ratio, Oral Sugar Test, and Insulin Response Test Results in Horses. *J Vet Intern Med,* 30**,** 1726-1731.
BEYTHIEN, E., WULF, M., ILLE, N., AURICH, J. & AURICH, C. 2017. Effects of sex, pregnancy and season on insulin secretion and carbohydrate metabolism in horses. *Anim Reprod Sci,* 184**,** 86-93.
BLAND, J. M. & ALTMAN, D. G. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet,* 1**,** 307-310.
BORER-WEIR, K. E., BAILEY, S. R., MENZIES-GOW, N. J., HARRIS, P. A. & ELLIOTT, J. 2012. Evaluation of a commercially available radioimmunoassay and species-specific ELISAs for measurement of high concentrations of insulin in equine serum. *Am J Vet Res,* 73**,** 1596-602.
BORER, K. E., BAILEY, S. R., MENZIES-GOW, N. J., HARRIS, P. A. & ELLIOTT, J. 2012. Effect of feeding glucose, fructose, and inulin on blood glucose and insulin concentrations in normal ponies and those predisposed to laminitis. *J Anim Sci,* 90**,** 3003-11.
BORGIA, L., VALBERG, S., MCCUE, M., WATTS, K. & PAGAN, J. 2011. Glycaemic and insulinaemic responses to feeding hay with different non-structural carbohydrate content in control and polysaccharide storage myopathy-affected horses. *J Anim Physiol Anim Nutr (Berl),* 95**,** 798-807.
BROJER, J., LINDASE, S., HEDENSKOG, J., ALVARSSON, K. & NOSTELL, K. 2013. Repeatability of the Combined Glucose-Insulin Tolerance Test and the Effect of a Stressor before Testing in Horses of 2 Breeds. *J Vet Intern Med*.
BUFF, P. R., DODDS, A. C., MORRISON, C. D., WHITLEY, N. C., MCFADIN, E. L., DANIEL, J. A., DJIANE, J. & KEISLER, D. H. 2002. Leptin in horses: tissue localization and relationship between peripheral concentrations of leptin and body condition. *J Anim Sci,* 80**,** 2942-8.
BURNS, T. A., GEOR, R. J., MUDGE, M. C., MCCUTCHEON, L. J., HINCHCLIFF, K. W. & BELKNAP, J. K. 2010. Proinflammatory cytokine and chemokine gene expression profiles in subcutaneous and visceral adipose tissue depots of insulin-resistant and insulin-sensitive light breed horses. *J Vet Intern Med,* 24**,** 932-9.
CALTABILOTA, T. J., EARL, L. R., THOMPSON, D. L., JR., CLAVIER, S. E. & MITCHAM, P. B. 2010. Hyperleptinemia in mares and geldings: assessment of insulin sensitivity from glucose responses to insulin injection. *J Anim Sci,* 88**,** 2940-9.
CAMPBELL, J. E. & DRUCKER, D. J. 2013. Pharmacology, physiology, and mechanisms of incretin hormone action. *Cell Metab,* 17**,** 819-37.
CARROLL, C. L. & HUNTINGTON, P. J. 1988. Body condition scoring and weight estimation of horses. *Equine Vet J,* 20**,** 41-45.
CARTER, R. A., GEOR, R. J., BURTON STANIAR, W., CUBITT, T. A. & HARRIS, P. A. 2009a. Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies. *Vet J,* 179**,** 204-10.
CARTER, R. A., MCCUTCHEON, L. J., GEORGE, L. A., SMITH, T. L., FRANK, N. & GEOR, R. J. 2009b. Effects of diet-induced weight gain on insulin sensitivity and plasma hormone and lipid concentrations in horses. *Am J Vet Res,* 70**,** 1250-8.
CARTER, R. A., TREIBER, K. H., GEOR, R. J., DOUGLASS, L. & HARRIS, P. A. 2009c. Prediction of incipient pasture-associated laminitis from hyperinsulinaemia, hyperleptinaemia and generalised and localised obesity in a cohort of ponies. *Equine Vet J,* 41**,** 171-178.

CAUGHEY, A. B. & TURRENTINE, M. 2018. ACOG Practice Bulletin No. 190 Summary: Gestational Diabetes Mellitus. *Obstet Gynecol,* 131**,** 406-408.

CEFALU, W. T. 2001. Insulin resistance: cellular and clinical concepts. *Exp Biol Med (Maywood),* 226**,** 13-26.

CHAMEROY, K. A., FRANK, N., ELLIOTT, S. B. & BOSTON, R. C. 2016. Comparison of Plasma Active Glucagon-Like Peptide 1 Concentrations in Normal Horses and Those With Equine Metabolic Syndrome and in Horses Placed on a High-Grain Diet. *J Equine Vet Sci,* 40**,** 16-25.

CHAMEROY, K. A., FRANK, N. & SCHUVER, A. 2010. Validation of an enzyme-linked immunosorbent assay (ELISA) for measurement of active glucagon-like peptide 1 (GLP-1) in equine plasma. *J Vet Intern Med,* 24**,** 780.

COFFMAN, J. R. & COLLES, C. M. 1983. Insulin tolerance in laminitic ponies. *Can J Comp Med,* 47**,** 347-51.

COLLICUTT, N. B., GARNER, B., BERGHAUS, R. D., CAMUS, M. S. & HART, K. 2015. Effect of delayed serum separation and storage temperature on serum glucose concentration in horse, dog, alpaca, and sturgeon. *Vet Clin Pathol,* 44**,** 120-7.

CONLON, J. M. 2001. Evolution of the insulin molecule: insights into structure-activity and phylogenetic relationships. *Peptides,* 22**,** 1183-93.

CORSON, D. C., WAGHORN, G. C., ULYATT, M. J. & LEE, J. 1999. NIRS: Forage and livestock feeding. *Proceedings of the New Zealand Grassland Association,* 61**,** 127-132.

D' FONSECA, N. M. M., GIBSON, C. M. E., VAN DOORN, D. A., DE RUIJTER-VILLANI, M., STOUT, T. A. E. & ROELFSEMA, E. 2020. Effect of long-term overfeeding of a high-energy diet on glucose tolerance in Shetland pony mares. *J Vet Intern Med,* 34**,** 1339-1349.

D'FONSECA, N. M. M., BEUKERS, M., WIJNBERG, I. D., NAVAS DE SOLIS, C., DE RUIJTER-VILLANI, M., VAN DOORN, D. A., STOUT, T. A. E. & ROELFSEMA, E. 2021. Effect of a long-term high-energy diet on cardiovascular parameters in Shetland pony mares. *J Vet Intern Med,* 35**,** 2427-2436.

DE GRAAF-ROELFSEMA, E. 2014. Glucose homeostasis and the enteroinsular axis in the horse: A possible role in equine metabolic syndrome. *Vet J,* 199**,** 11-18.

DE GRAAF-ROELFSEMA, E., VAN GINNEKEN, M. E., VAN BREDA, E., WIJNBERG, I. D., KEIZER, H. A. & VAN DER KOLK, J. H. 2006. The effect of long-term exercise on glucose metabolism and peripheral insulin sensitivity in standardbred horses. *Equine Vet J Suppl***,** 221-5.

DE LAAT, M. A., FITZGERALD, D. M., SILLENCE, M. N. & SPENCE, R. J. 2018. Glucagon-like peptide-2: A potential role in equine insulin dysregulation. *Equine Vet J,* 50**,** 842-847.

DE LAAT, M. A., MCGOWAN, C. M., SILLENCE, M. N. & POLLITT, C. C. 2010. Equine laminitis: Induced by 48 h hyperinsulinaemia in Standardbred horses. *Equine Vet J,* 42**,** 129-35.

DE LAAT, M. A., MCGREE, J. M. & SILLENCE, M. N. 2016. Equine hyperinsulinemia: investigation of the enteroinsular axis during insulin dysregulation. *Am J Physiol Endocrinol Metab,* 310**,** E61-72.

DE LAAT, M. A., POLLITT, C. C., KYAW-TANNER, M. T., MCGOWAN, C. M. & SILLENCE, M. N. 2013. A potential role for lamellar insulin-like growth factor-1 receptor in the pathogenesis of hyperinsulinaemic laminitis. *Vet J,* 197**,** 302-6.

DE LAAT, M. A. & SILLENCE, M. N. 2017. The repeatability of an oral glucose test in ponies. *Equine Vet J,* 49**,** 238-243.

DEMING, W. E. 1943. *Statistical adjustment of data,* New York, John Wiley and Sons.

DIAKOGIANNAKI, E., GRIBBLE, F. M. & REIMANN, F. 2012. Nutrient detection by incretin hormone secreting cells. *Physiol Behav,* 106**,** 387-93.

DRAZNIN, B. 2006. Molecular mechanisms of insulin resistance: serine phosphorylation of insulin receptor substrate-1 and increased expression of p85alpha: the two sides of a coin. *Diabetes,* 55**,** 2392-7.
DRUCKER, D. J., BOUSHEY, R. P., WANG, F., HILL, M. E., BRUBAKER, P. L. & YUSTA, B. 1999. Biologic properties and therapeutic potential of glucagon-like peptide-2. *JPEN J Parenter Enteral Nutr,* 23**,** S98-100.
DUEHLMEIER, R., DEEGAN, E., FUHRMANN, H., WIDDEL, A. & SALLMANN, H. P. 2001. Glucose-dependent insulinotropic polypeptide (GIP) and the enteroinsular axis in equines (*Equus caballus)*. *Comparative Biochemistry and Physiology Part A,* 129**,** 563-575.
DUGDALE, A. H., CURTIS, G. C., CRIPPS, P., HARRIS, P. A. & ARGO, C. M. 2010. Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares. *Equine Vet J,* 42**,** 600-10.
DUGDALE, A. H., GROVE-WHITE, D., CURTIS, G. C., HARRIS, P. A. & ARGO, C. M. 2012. Body condition scoring as a predictor of body fat in horses and ponies. *Vet J,* 194**,** 173-8.
DUHLMEIER, R., DEEGAN, E., FUHRMANN, H., WIDDEL, A. & SALLMANN, H. P. 2001. Glucose dependent insulinotropic polypeptide (GIP) and the enteroinsular axis in equines (Equus caballus). *Comparative Biochemistry and Physiology Part A,* 129**,** 563-575.
DUNBAR, L. K., MIELNICKI, K. A., DEMBEK, K. A., TORIBIO, R. E. & BURNS, T. A. 2016. Evaluation of Four Diagnostic Tests for Insulin Dysregulation in Adult Light-Breed Horses. *J Vet Intern Med,* 30**,** 885-91.
DURHAM, A. E. 2017. Therapeutics for Equine Endocrine Disorders. *Vet Clin North Am Equine Pract,* 33**,** 127-139.
DURHAM, A. E., FRANK, N., MCGOWAN, C. M., MENZIES-GOW, N. J., ROELFSEMA, E., VERVUERT, I., FEIGE, K. & FEY, K. 2019. ECEIM consensus statement on equine metabolic syndrome. *J Vet Intern Med,* 33**,** 335-349.
DURHAM, A. E., MCGOWAN, C. M., FEY, K., TAMZALI, Y. & VAN DER KOLK, J. H. 2014. Pituitarypars intermediadysfunction: Diagnosis and treatment. *Equine Veterinary Education,* 26**,** 216-223.
DUTTON, D. W., LASHNITS, K. J. & WEGNER, K. 2009. Managing severe hoof pain in a horse using multimodal analgesia and a modified composite pain score. *Equine Veterinary Education,* 21**,** 37-43.
EILER, H., FRANK, N., ANDREWS, F. M., OLIVER, J. W. & FECTEAU, K. A. 2005. Physiologic assessment of blood glucose homeostasis via combined intravenous glucose and insulin testing in horses. *Am J Vet Res,* 66**,** 1598-604.
ESSER, N., LEGRAND-POELS, S., PIETTE, J., SCHEEN, A. J. & PAQUOT, N. 2014. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Res Clin Pract,* 105**,** 141-50.
FITZGERALD, D. M., ANDERSON, S. T., SILLENCE, M. N. & DE LAAT, M. A. 2019a. The cresty neck score is an independent predictor of insulin dysregulation in ponies. *PLoS One,* 14**,** e0220203.
FITZGERALD, D. M., WALSH, D. M., SILLENCE, M. N., POLLITT, C. C. & DE LAAT, M. A. 2019b. Insulin and incretin responses to grazing in insulin-dysregulated and healthy ponies. *J Vet Intern Med,* 33**,** 225-232.
FOWDEN, A. L., COMLINE, R. S. & SILVER, M. 1984. Insulin secretion and carbohydrate metabolism during pregnancy in the mare. *Equine Vet J,* 16**,** 239-46.
FRANK, N., BAILEY, S. R., BERTIN, F. R., DE LAAT, M., DURHAM, A., KRITCHEVSKY, J. & MENZIES-GOW, N. 2020. *Recommendations for the Diagnosis and Treatment of Equine Metabolic Syndrome (EMS)* [Online]. Available: https://sites.tufts.edu/equineendogroup/files/2020/09/200592_EMS_Recommendations_Bro-FINAL.pdf [Accessed].

FRANK, N. & GEOR, R. 2014. Current best practice in clinical management of equine endocrine patients. *Equine Veterinary Education,* 26**,** 6-9.
FRANK, N., GEOR, R. J., BAILEY, S. R., DURHAM, A. E., JOHNSON, P. J. & AMERICAN COLLEGE OF VETERINARY INTERNAL, M. 2010. Equine metabolic syndrome. *J Vet Intern Med,* 24**,** 467-75.
FRANK, N., SOJKA, J. & MESSER, N. T. T. 2002. Equine thyroid dysfunction. *Vet Clin North Am Equine Pract,* 18**,** 305-19, vii.
FRANK, N. & TADROS, E. M. 2014. Insulin dysregulation. *Equine Vet J,* 46**,** 103-12.
FRANK, N. & WALSH, D. M. 2017. Repeatability of Oral Sugar Test Results, Glucagon-Like Peptide-1 Measurements, and Serum High-Molecular-Weight Adiponectin Concentrations in Horses. *J Vet Intern Med*.
FREESTONE, J. F., BEADLE, K., SHOEMAKER, R. T., BESSIN, R. T., WOLFSHEIMER, K. J. & CHURCH, C. 1992. Improved insulin sensitivity in hyperinsulinaemic ponies through physical conditioning and controlled feed intake. *Equine Vet J,* 24**,** 187-190.
FURTADO, T., PERKINS, E., PINCHBECK, G., MCGOWAN, C., WATKINS, F. & CHRISTLEY, R. 2021. Exploring horse owners' understanding of obese body condition and weight management in UK leisure horses. *Equine Vet J,* 53**,** 752-762.
GEOR, R. J. 2010. Current concepts on the pathophysiology of pasture-associated laminitis. *Vet Clin North Am Equine Pract,* 26**,** 265-76.
GILES, S. L., RANDS, S. A., NICOL, C. J. & HARRIS, P. A. 2014. Obesity prevalence and associated risk factors in outdoor living domestic horses and ponies. *PeerJ,* 2**,** e299.
GODSLAND, I. F. 2005. Oestrogens and insulin secretion. *Diabetologia,* 48**,** 2213-2220.
GOOSSENS, G. H. 2008. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. *Physiol Behav,* 94**,** 206-18.
GORDON, M. E., MCKEEVER, K. H., BETROS, C. L. & MANSO FILHO, H. C. 2007a. Exercise-induced alterations in plasma concentrations of ghrelin, adiponectin, leptin, glucose, insulin, and cortisol in horses. *Vet J,* 173**,** 532-40.
GORDON, M. E., MCKEEVER, K. H., BETROS, C. L. & MANSO FILHO, H. C. 2007b. Plasma leptin, ghrelin and adiponectin concentrations in young fit racehorses versus mature unfit standardbreds. *Vet J,* 173**,** 91-100.
GRAVES, E. A., SCHOTT, H. C., JOHNSON, P. J., SLIGHT, S. H., MESSER, N. T., GANJAM, V. K., EBERHART, S., REFSAL, K. R., NACHREINER, R. & NICKELS, F. Thyroid function in horses with Peripheral Cushing's syndrome Annual convention of the American Association of Equien Practitioners, 2002 Orlando, USA. 178-180.
GRILL, V. & QVIGSTAD, E. 2000. Fatty acids and insulin secretion. *Br J Nutr,* 83 Suppl 1**,** S79-84.
HACKETT, E. S. & MCCUE, P. M. 2010. Evaluation of a veterinary glucometer for use in horses. *J Vet Intern Med,* 24**,** 617-21.
HAMPTON, S. M., MORGAN, L. M., TREDGER, J. A., CRAMB, R. & MARKS, V. 1986. Insulin and C-peptide levels after oral and intravenous glucose. Contribution of enteroinsular axis to insulin secretion. *Diabetes,* 35**,** 612-6.
HANSEN, L., DEACON, C. F., ORSKOV, C. & HOLST, J. J. 1999. Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology,* 140**,** 5356-63.
HARRIS, P. A., NELSON, S., CARSLAKE, H. B., ARGO, C. M., WOLF, R., FABRI, F. B., BROLSMA, K. M., VAN OOSTRUM, M. J. & ELLIS, A. D. 2018. Comparison of NIRS and Wet Chemistry Methods for the Nutritional Analysis of Haylages for Horses. *J Equine Vet Sci,* 71**,** 13-20.
HART, K. A., WOCHELE, D. M., NORTON, N. A., MCFARLANE, D., WOOLDRIDGE, A. A. & FRANK, N. 2016. Effect of Age, Season, Body Condition, and Endocrine Status on

Serum Free Cortisol Fraction and Insulin Concentration in Horses. *J Vet Intern Med,* 30**,** 653-63.
HERBERT, V., LAU, K. S., GOTTLIEB, C. W. & BLEICHER, S. J. 1965. Coated charcoal immunoassay of insulin. *J Clin Endocrinol Metab,* 25**,** 1375-84.
HERRMANN, C., GÖKE, R., RICHTER, G., FEHMANN, H. C., ARNOLD, R. & GÖKE, B. 1995. Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients. *Digestion,* 56**,** 117-26.
HO, E. N., WAN, T. S., WONG, A. S., LAM, K. K. & STEWART, B. D. 2008. Doping control analysis of insulin and its analogues in equine plasma by liquid chromatography-tandem mass spectrometry. *J Chromatogr A,* 1201**,** 183-90.
HO, E. N., WAN, T. S., WONG, A. S., LAM, K. K. & STEWART, B. D. 2011. Doping control analysis of insulin and its analogues in equine urine by liquid chromatography-tandem mass spectrometry. *J Chromatogr A,* 1218**,** 1139-46.
HOFFMAN, R. M., BOSTON, R. C., STEFANOVSKI, D., KRONFELD, D. S. & HARRIS, P. A. 2003. Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred Geldings. *J Anim Sci,* 81**,** 2333-2342.
HOLST, J. J. 2019. The incretin system in healthy humans: The role of GIP and GLP-1. *Metabolism - Clinical and Experimental,* 96**,** 46-55.
HOSMER, D. W. & LEMESHOW, S. 2005. Model-building strategies and methods for logistic regression. *In:* CRESSIE, N. A. C., FISHER, N. I. & JOHNSTONE, I. M. (eds.) *Applied Logistic Regression.* 2nd ed. New York: John Wiley & Sons.
JAKOBSEN, J. C., GLUUD, C., WETTERSLEV, J. & WINKEL, P. 2017. When and how should multiple imputation be used for handling missing data in randomised clinical trials – a practical guide with flowcharts. *BMC Medical Research Methodology,* 17**,** 162.
JEFFCOTT, L. B., FIELD, J. R., MCLEAN, J. G. & O'DEA, K. 1986. Glucose tolerance and insulin sensitivity in ponies and Standardbred horses. *Equine Vet J,* 18**,** 97-101.
JOCELYN, N. A., HARRIS, P. A. & MENZIES-GOW, N. J. 2018. Effect of varying the dose of corn syrup on the insulin and glucose response to the oral sugar test. *Equine Vet J,* 50**,** 836-841.
JOHNSON, P. J. 2002. The equine metabolic syndrome Peripheral Cushing's syndrome. *Vet Clin North Am Equine Pract,* 18**,** 271-293.
JOHNSON, P. J., SLIGHT, S. H. & GANJAM, V. K. Laminitis, "hypothyroidism" and obesity: a peripheral Cushingoid syndrome in horses? Annual Forum of the ACVIM, 1999 Lakewood (CO), USA. 192-194.
JOHNSON, P. J., WIEDMEYER, C. E., MESSER, N. T. & GANJAM, V. K. 2009. Medical implications of obesity in horses - lessons for human obesity. *J Diabetes Sci Technol,* 3**,** 163-74.
KARAKELIDES, H., IRVING, B. A., SHORT, K. R., O'BRIEN, P. & NAIR, K. S. 2010. Age, obesity, and sex effects on insulin sensitivity and skeletal muscle mitochondrial function. *Diabetes,* 59**,** 89-97.
KARIKOSKI, N. P., HORN, I., MCGOWAN, T. W. & MCGOWAN, C. M. 2011. The prevalence of endocrinopathic laminitis among horses presented for laminitis at a first-opinion/referral equine hospital. *Domest Anim Endocrinol,* 41**,** 111-7.
KARIKOSKI, N. P., MCGOWAN, C. M., SINGER, E. R., ASPLIN, K. E., TULAMO, R. M. & PATTERSON-KANE, J. C. 2015. Pathology of Natural Cases of Equine Endocrinopathic Laminitis Associated With Hyperinsulinemia. *Vet Pathol,* 52**,** 945-56.
KEARNS, C. F., MCKEEVER, K. H., ROEGNER, V., BRADY, S. M. & MALINOWSKI, K. 2006. Adiponectin and leptin are related to fat mass in horses. *Vet J,* 172**,** 460-5.
KHEDER, M. H., BAILEY, S. R., DUDLEY, K. J., SILLENCE, M. N. & DE LAAT, M. A. 2018. Equine glucagon-like peptide-1 receptor physiology. *PeerJ,* 6**,** e4316.

KIM, J. A., MONTAGNANI, M., KOH, K. K. & QUON, M. J. 2006. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation,* 113**,** 1888-904.

KNOWLES, E. J. & GRIEVE, L. 2020. Clinical insights: Equine obesity. *Equine Vet J,* 52**,** 635-638.

KNOWLES, E. J., HARRIS, P. A., ELLIOTT, J. & MENZIES-GOW, N. J. 2016. Use of the oral sugar test in ponies when performed with or without prior fasting. *Equine Vet J*.

KNOWLES, E. J., WITHERS, J. M. & MAIR, T. S. 2012. Increased plasma fructosamine concentrations in laminitic horses. *Equine Vet J,* 44**,** 226-9.

KOHNKE, J. 1992. *Feeding and Nutrition: The Making of a Champion,* Australia, Birubi Pacific.

KÖLLER, G., BASSEWITZ, K. & SCHUSSER, G. F. 2016. Reference ranges of insulin, insulin like growth factor-1 and adrenocorticotropic hormone in ponies. *Tierarztl Prax Ausg G Grosstiere Nutztiere,* 44**,** 19-25.

KRONFELD, D. S., TREIBER, K. H. & GEOR, R. J. 2005. Comparison of nonspecific indications and quantitative methods for the assessment of insulin resistance in horses and ponies. *J Am Vet Med Assoc,* 226**,** 712-9.

LANDIS, J. R. & KOCH, G. G. 1977. The measurement of observer agreement for categorical data. *Biometrics,* 33**,** 159-74.

LESCHKE, D. H., MUIR, G. S., HODGSON, J. K., COYLE, M., HORN, R. & BERTIN, F.-R. 2019. Immunoreactive insulin stability in horses at risk of insulin dysregulation. *J Vet Intern Med,* 33**,** 2746-2751.

LEWIS, S. L., HOLL, H. M., STREETER, C., POSBERGH, C., SCHANBACHER, B. J., PLACE, N. J., MALLICOTE, M. F., LONG, M. T. & BROOKS, S. A. 2017. Genomewide association study reveals a risk locus for equine metabolic syndrome in the Arabian horse. *J Anim Sci,* 95**,** 1071-1079.

LINDASE, S., MULLER, C., NOSTELL, K. & BROJER, J. 2018. Evaluation of glucose and insulin response to haylage diets with different content of nonstructural carbohydrates in 2 breeds of horses. *Domest Anim Endocrinol,* 64**,** 49-58.

LINDÅSE, S., NOSTELL, K., BERGSTEN, P., FORSLUND, A. & BRÖJER, J. 2021. Evaluation of fasting plasma insulin and proxy measurements to assess insulin sensitivity in horses. *BMC Vet Res,* 17**,** 78.

LINDASE, S., NOSTELL, K., SODER, J. & BROJER, J. 2017. Relationship Between beta-cell Response and Insulin Sensitivity in Horses based on the Oral Sugar Test and the Euglycemic Hyperinsulinemic Clamp. *J Vet Intern Med,* 31**,** 1541-1550.

LONGLAND, A. C., BARFOOT, C. & HARRIS, P. A. 2011. Effects of soaking on the water-soluble carbohydrate and crude protein content of hay. *Vet Rec,* 168**,** 618.

LONGLAND, A. C., BARFOOT, C. & HARRIS, P. A. 2014. Effect of period, water temperature and agitation on loss of water-soluble carbohydrates and protein from grass hay: implications for equine feeding management. *Vet Rec,* 174**,** 68.

LOOS, C. M. M., DORSCH, S. C., ELZINGA, S. E., BREWSTER-BARNES, T., VANZANT, E. S., ADAMS, A. A. & URSCHEL, K. L. 2019. A high protein meal affects plasma insulin concentrations and amino acid metabolism in horses with equine metabolic syndrome. *Vet J,* 251**,** 105341.

MACK, S. J., DUGDALE, A. H., ARGO, C. M., MORGAN, R. A. & MCGOWAN, C. M. 2014. Impact of water-soaking on the nutrient composition of UK hays. *Vet Rec,* 174**,** 452.

MALAISSE, W. J. 1997. Insulin biosynthesis and secretion *in vitro*. *In:* K., A., ZIMMET, P. & DEFRONZO, R. (eds.) *International textbook of diabetes.* 2nd ed. New York: John Wiley and Sons.

MCGOWAN, C. M., DUGDALE, A. H. & ARGO, C. M. 2014. Effect of feeding soaked hay for two weeks on dynamic endocrine testing in horses. *European Equine Endocrinology Symposium.* Windsor, UK.
MCGOWAN, C. M., DUGDALE, A. H., PINCHBECK, G. L. & ARGO, C. M. 2013a. Dietary restriction in combination with a nutraceutical supplement for the management of equine metabolic syndrome in horses. *Vet J,* 196**,** 153-9.
MCGOWAN, T., GEOR, R., EVANS, H., SILLENCE, M., MUNN, K. & MCGOWAN, C. 2008. Comparison of 4 assays for serum insulin analysis in the horse (abstract). *J Vet Intern Med,* 22**,** 734-735.
MCGOWAN, T. W., PINCHBECK, G. P. & MCGOWAN, C. M. 2013b. Prevalence, risk factors and clinical signs predictive for equine pituitary pars intermedia dysfunction in aged horses. *Equine Vet J,* 45**,** 74-9.
MEIER, A. D., DE LAAT, M. A., REICHE, D. B., POLLITT, C. C., WALSH, D. M., MCGREE, J. M. & SILLENCE, M. N. 2018. The oral glucose test predicts laminitis risk in ponies fed a diet high in nonstructural carbohydrates. *Domest Anim Endocrinol,* 63**,** 1-9.
MEIER, A. D., DE LAAT, M. A., REICHE, D. B. & SILLENCE, M. N. 2020. Glucagon-like peptide-1, insulin-like growth factor-1, and adiponectin in insulin-dysregulated ponies: effects of feeding a high nonstructural carbohydrate diet and association with prospective laminitis. *Domest Anim Endocrinol,* 71**,** 106397.
MENZIES-GOW, N. J., HARRIS, P. A. & ELLIOTT, J. 2017. Prospective cohort study evaluating risk factors for the development of pasture-associated laminitis in the United Kingdom. *Equine Vet J,* 49**,** 300-306.
MENZIES-GOW, N. J., KNOWLES, E. J., ROGERS, I. & RENDLE, D. I. 2018. Validity and application of immunoturbidimetric and enzyme-linked immunosorbent assays for the measurement of adiponectin concentration in ponies. *Equine Vet J*.
MERCODIA 2016. Mercodia Equine Insulin ELISA. Directions for Use Version 4.0. https://www.mercodia.se/assets/upload/files/DfU/Equine/Insulin%20Equine%20version%204_0.pdf
MILLIPORE 2012. ELISA Kit High Sensitivity GLP-1 Protocol.
MOORE, M., FERGUSON, J., RIGSBY, P. & BURNS, C. 2019a. Proposed 1st WHO International Standard for insulin, human. Geneva.
MOORE, M., FERNGUSON, J., RIGSBY, P. & BURNS, C. 2019b. Proposed 1st WHO international standard for insulin, human. https://www.who.int/biologicals/expert_committee/BS.2019.2366_insulin_final.pdf: World Health organisation Expert Committee on Biologiical Standardization.
MORGAN, K. 1998. Thermoneutral Zone and Critical Temperatures of Horses. *Journal of Thermal Biology,* 23**,** 59-61.
MORGAN, R., KEEN, J. & MCGOWAN, C. 2015. Equine metabolic syndrome. *Vet Rec,* 177**,** 173-9.
MORGAN, R., TEO, D. & KIRTON, R. 2020. The equine pancreas in chronic hyperinsulinaemia. *4th Global Equine Endocrine Symposium* Bavaria, Germany.
MORGAN, R. A., KEEN, J. A. & MCGOWAN, C. M. 2016a. Treatment of equine metabolic syndrome: A clinical case series. *Equine Vet J,* 48**,** 422-6.
MORGAN, R. A., KEEN, J. A., WALKER, B. R. & HADOKE, P. W. 2016b. Vascular Dysfunction in Horses with Endocrinopathic Laminitis. *PLoS One,* 11**,** e0163815.
MORGAN, R. A., MCGOWAN, T. W. & MCGOWAN, C. M. 2014. Prevalence and risk factors for hyperinsulinaemia in ponies in Queensland, Australia. *Aust Vet J,* 92**,** 101-6.
MÜLLER, C. E. 2009. Long-stemmed vs. cut haylage in bales—Effects on fermentation, aerobic storage stability, equine eating behaviour and characteristics of equine faeces. *Animal Feed Science and Technology,* 152**,** 307-321.

MÜLLER, C. E. 2012. Equine digestion of diets based on haylage harvested at different plant maturities. *Animal Feed Science and Technology,* 177**,** 65-74.
MÜLLER, C. E. & UDÉN, P. 2007. Preference of horses for grass conserved as hay, haylage or silage. *Animal Feed Science and Technology,* 132**,** 66-78.
NAJJAR, S. & PERDOMO, G. 2019. Hepatic Insulin Clearance: Mechanism and Physiology. *Physiology,* 34**,** 198-215.
NANAYAKKARA, S. N., RAHNAMA, S., HARRIS, P. A., ANDERSON, S. T., DE LAAT, M. A., BAILEY, S. & SILLENCE, M. N. 2019. Characterization of insulin and IGF-1 receptor binding in equine liver and lamellar tissue: implications for endocrinopathic laminitis. *Domest Anim Endocrinol,* 66**,** 21-26.
NAUCK, M. 2016. Incretin therapies: highlighting common features and differences in the modes of action of glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors. *Diabetes Obes Metab,* 18**,** 203-16.
NEWKIRK, K. M., EHRENSING, G., ODOI, A., BOSTON, R. C. & FRANK, N. 2018. Immunohistochemical expression of insulin, glucagon, and somatostatin in pancreatic islets of horses with and without insulin resistance. *Am J Vet Res,* 79**,** 191-198.
NISWENDER, K. D. 2011. Basal insulin: physiology, pharmacology, and clinical implications. *Postgrad Med,* 123**,** 17-26.
NORTON, E. M., AVILA, F., SCHULTZ, N. E., MICKELSON, J. R., GEOR, R. J. & MCCUE, M. E. 2019a. Evaluation of an HMGA2 variant for pleiotropic effects on height and metabolic traits in ponies. *J Vet Intern Med,* 33**,** 942-952.
NORTON, E. M., SCHULTZ, N. E., RENDAHL, A. K., MCFARLANE, D., GEOR, R. J., MICKELSON, J. R. & MCCUE, M. E. 2019b. Heritability of metabolic traits associated with equine metabolic syndrome in Welsh ponies and Morgan horses. *Equine Vet J,* 51**,** 475-480.
OBEL, N. 1948. *Studies on the histopathology of acute laminitis.* Uppsala, Sweden.
ÖBERG, J., BRÖJER, J., WATTLE, O. & LILLIEHÖÖK, I. 2011. Evaluation of an equine-optimized enzyme-linked immunosorbent assay for serum insulin measurement and stability study of equine serum insulin. *Comparative Clinical Pathology,* 21**,** 1291-1300.
ODDOZE, C., LOMBARD, E. & PORTUGAL, H. 2012. Stability study of 81 analytes in human whole blood, in serum and in plasma. *Clin Biochem,* 45**,** 464-9.
MET OFFICE. 2016. *Northwest England and Isle of Man: Climate* [Online]. Available: https://www.metoffice.gov.uk/binaries/content/assets/metofficegovuk/pdf/weather/learn-about/uk-past-events/regional-climates/north-west-england--isle-of-man_-climate---met-office.pdf [Accessed 7th February 2020].
OLLEY, R. B., CARSLAKE, H. B., IRELAND, J. L. & MCGOWAN, C. M. 2019. Comparison of fasted basal insulin with the combined glucose-insulin test in horses and ponies with suspected insulin dysregulation. *Vet J,* 252**,** 105351.
OWEN, W. E. & ROBERTS, W. L. 2004. Cross-reactivity of three recombinant insulin analogs with five commercial insulin immunoassays. *Clin Chem,* 50**,** 257-9.
OWERS, R. & CHUBBOCK, S. 2013. Fight the fat! *Equine Vet J,* 45**,** 5.
PAGAN, J. D. 1998. Measuring the Digestible Energy Content of Horse Feeds. *In:* PAGAN, J. D. (ed.) *Advances in Equine Nutrition.* Nottingham, U.K.: Nottingham University Press.
PARK, J. Y. & KRICKA, L. J. 2013. Chapter 5.3 - Interferences in Immunoassay. *In:* WILD, D. (ed.) *The Immunoassay Handbook (Fourth Edition).* Oxford: Elsevier.
PERKINS, G. A., LAMB, S., ERB, H. N., SCHANBACHER, B., NYDAM, D. V. & DIVERS, T. J. 2002. Plasma adrenocorticotropin (ACTH) concentrations and clinical response in horses treated for equine Cushing's disease with cyproheptadine or pergolide. *Equine Vet J,* 34**,** 679-85.

PETERSEN, M. C. & SHULMAN, G. I. 2018. Mechanisms of Insulin Action and Insulin Resistance. *Physiol Rev,* 98**,** 2133-2223.

PLACE, N. J., MCGOWAN, C. M., LAMB, S. V., SCHANBACHER, B. J., MCGOWAN, T. & WALSH, D. M. 2010. Seasonal variation in serum concentrations of selected metabolic hormones in horses. *J Vet Intern Med,* 24**,** 650-4.

PLEASANT, R. S., SUAGEE, J. K., THATCHER, C. D., ELVINGER, F. & GEOR, R. J. 2013. Adiposity, plasma insulin, leptin, lipids, and oxidative stress in mature light breed horses. *J Vet Intern Med,* 27**,** 576-82.

POLLARD, D., WYLIE, C. E., NEWTON, J. R. & VERHEYEN, K. L. P. 2019a. Incidence and clinical signs of owner-reported equine laminitis in a cohort of horses and ponies in Great Britain. *Equine Vet J,* 51**,** 587-594.

POLLARD, D., WYLIE, C. E., VERHEYEN, K. L. P. & NEWTON, J. R. 2019b. Identification of modifiable factors associated with owner-reported equine laminitis in Britain using a web-based cohort study approach. *BMC Vet Res,* 15**,** 59.

POLLEX, R. L. & HEGELE, R. A. 2006. Genetic determinants of the metabolic syndrome. *Nat Clin Pract Cardiovasc Med,* 3**,** 482-9.

POWELL, D. M., REEDY, S. E., SESSIONS, D. R. & FITZGERALD, B. P. 2002. Effect of short-term exercise training on insulin sensitivity in obese and lean mares. *Equine Vet J Suppl***,** 81-4.

PRATT-PHILLIPS, S. E., GEOR, R. J. & MCCUTCHEON, L. J. 2015. Comparison among the euglycemic-hyperinsulinemic clamp, insulin-modified frequently sampled intravenous glucose tolerance test, and oral glucose tolerance test for assessment of insulin sensitivity in healthy Standardbreds. *Am J Vet Res,* 76**,** 84-91.

PRUTTON, J. S., KASS, P. H., WATSON, J. L. & PUSTERLA, N. 2015. Pre-analytical stability of adrenocorticotrophic hormone from healthy horses in whole blood, plasma and frozen plasma samples. *Vet J,* 204**,** 123-4.

RAGNO, V. M., KLEIN, C. D., SEREDA, N. S., UEHLINGER, F. D., ZELLO, G. A., ROBINSON, K. A. & MONTGOMERY, J. B. 2021. Morphometric, metabolic, and inflammatory markers across a cohort of client-owned horses and ponies on the insulin dysregulation spectrum. *J Equine Vet Sci,* 105**,** 103715.

RAPSON, J. L., SCHOTT, H. C., 2ND, NIELSEN, B. D., MCCUTCHEON, L. J., HARRIS, P. A. & GEOR, R. J. 2018. Effects of age and diet on glucose and insulin dynamics in the horse. *Equine Vet J,* 50**,** 690-696.

RASBACH, J., STEELE, F., BROWNE, W. K. & GOLDSTEIN, H. 2017. A User's Guide to MLwiN, v3.01. *In:* CENTRE FOR MULTILEVEL MODELLING, U. O. B. (ed.).

REIMERS, T. J., COWAN, R. G., MCCANN, J. P. & ROSS, M. W. 1982. Validation of a rapid solid-phase radioimmunoassay for canine, bovine, and equine insulin. *Am J Vet Res,* 43**,** 1274-8.

RENDLE, D., ARGO, C. M., BOWEN, M., CARSLAKE, H., GERMAN, A., HARRIS, P., KNOWLES, E. J., MENZIES-GOW, N. & MORGAN, R. 2018. Equine obesity: current perspectives. *UK-Vet Equine,* 2**,** 1-19.

RENDLE, D. I., HELLER, J., HUGHES, K. J., INNOCENT, G. T. & DURHAM, A. E. 2009. Stability of common biochemistry analytes in equine blood stored at room temperature. *Equine Vet J,* 41**,** 428-32.

ROBIN, C. A., IRELAND, J. L., WYLIE, C. E., COLLINS, S. N., VERHEYEN, K. L. & NEWTON, J. R. 2015. Prevalence of and risk factors for equine obesity in Great Britain based on owner-reported body condition scores. *Equine Vet J,* 47**,** 196-201.

RODRIGUEZ-CABALEIRO, D., VAN UYTFANGHE, K., STOVE, V., FIERS, T. & THIENPONT, L. M. 2007. Pilot study for the standardization of insulin immunoassays with isotope dilution liquid chromatography/tandem mass spectrometry. *Clin Chem,* 53**,** 1462-9.

SCHOBER, P., BOER, C. & SCHWARTE, L. A. 2018. Correlation Coefficients: Appropriate Use and Interpretation. *Anesthesia & Analgesia,* 126**,** 1763-1768.

SCHOTT, H. C., ANDREWS, F. M., DURHAM, A., FRANK, N., HART, K. A., KRITCHEVSKY, J., MCFARLANE, D. & STEWART, A. A. 2019. *Recommendations for the Diagnosis and Treatment of PPID* [Online]. Available: https://sites.tufts.edu/equineendogroup/files/2019/12/2019-PPID_EEGbooklet.pdf [Accessed 10 February 2020].

SCHUVER, A., FRANK, N., CHAMEROY, K. A. & ELLIOTT, S. B. 2014. Assessment of Insulin and Glucose Dynamics by Using an Oral Sugar Test in Horses. *J Equine Vet Sci,* 34**,** 465-470.

SHANIK, M. H., XU, Y., SKRHA, J., DANKNER, R., ZICK, Y. & ROTH, J. 2008. Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? *Diabetes Care,* 31 Suppl 2**,** S262-8.

SMITH, S., HARRIS, P. A. & MENZIES-GOW, N. J. 2016. Comparison of the in-feed glucose test and the oral sugar test. *Equine Vet J,* 48**,** 224-7.

STATEN, M. A., STERN, M. P., MILLER, W. G., STEFFES, M. W., CAMPBELL, S. E. & INSULIN STANDARDIZATION, W. 2010. Insulin assay standardization: leading to measures of insulin sensitivity and secretion for practical clinical care. *Diabetes Care,* 33**,** 205-6.

STEPHENSON, H. M., GREEN, M. J. & FREEMAN, S. L. 2011. Prevalence of obesity in a population of horses in the UK. *Vet Rec,* 168**,** 131.

STEWART-HUNT, L., GEOR, R. J. & MCCUTCHEON, L. J. 2006. Effects of short-term training on insulin sensitivity and skeletal muscle glucose metabolism in standardbred horses. *Equine Vet J Suppl***,** 226-32.

SUAGEE, J. K., CORL, B. A., CRISMAN, M. V., HULVER, M. W., MCCUTCHEON, L. J. & GEOR, R. J. 2011. Effects of acute hyperinsulinemia on inflammatory proteins in horses. *Vet Immunol Immunopathol,* 142**,** 141-6.

SUAGEE, J. K., CORL, B. A. & GEOR, R. J. 2012. A Potential Role for Pro-Inflammatory Cytokines in the Development of Insulin Resistance in Horses. *Animals,* 2**,** 243-60.

TADROS, E. M., FOWLIE, J. G., REFSAL, K. R., MARTENIUK, J. & SCHOTT, H. C., 2ND 2018. Association between hyperinsulinaemia and laminitis severity at the time of pituitary pars intermedia dysfunction diagnosis. *Equine Vet J*.

TADROS, E. M., FRANK, N., DE WITTE, F. G. & BOSTON, R. C. 2013. Effects of intravenous lipopolysaccharide infusion on glucose and insulin dynamics in horses with equine metabolic syndrome. *Am J Vet Res,* 74**,** 1020-9.

THATCHER, C. D., PLEASANT, R. S., GEOR, R. J. & ELVINGER, F. 2012. Prevalence of overconditioning in mature horses in southwest Virginia during the summer. *J Vet Intern Med,* 26**,** 1413-8.

TINWORTH, K. D., WYNN, P. C., BOSTON, R. C., HARRIS, P. A., SILLENCE, M. N., THEVIS, M., THOMAS, A. & NOBLE, G. K. 2011. Evaluation of commercially available assays for the measurement of equine insulin. *Domest Anim Endocrinol,* 41**,** 81-90.

TINWORTH, K. D., WYNN, P. C., HARRIS, P. A., SILLENCE, M. N. & NOBLE, G. K. 2009. Optimising the Siemens Coat-A-Count Radioimmunoassay to Measure Insulin in Equine Plasma. *J Equine Vet Sci,* 29**,** 411-413.

TOTH, F., FRANK, N., CHAMEROY, K. A. & BOSTONT, R. C. 2009. Effects of endotoxaemia and carbohydrate overload on glucose and insulin dynamics and the development of laminitis in horses. *Equine Vet J,* 41**,** 852-8.

TOTH, F., FRANK, N., MARTIN-JIMENEZ, T., ELLIOTT, S. B., GEOR, R. J. & BOSTON, R. C. 2010. Measurement of C-peptide concentrations and responses to somatostatin, glucose infusion, and insulin resistance in horses. *Equine Vet J,* 42**,** 149-55.

TREIBER, K. H., KRONFELD, D. S., HESS, T. M., BOSTON, R. C. & HARRIS, P. A. 2005. Use of proxies and reference quintiles obtained from minimal model analysis for

determination of insulin sensitivity and pancreatic beta-cell responsiveness in horses. *Am J Vet Res,* 66**,** 2114-21.
TREIBER, K. H., KRONFELD, D. S., HESS, T. M., BYRD, B. M., SPLAN, R. K. & STANIAR, W. B. 2006. Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies. *J Am Vet Med Assoc,* 228**,** 1538-1545.
VENUGOPAL, C. S., EADES, S., HOLMES, E. P. & BEADLE, R. E. 2011. Insulin resistance in equine digital vessel rings: an in vitro model to study vascular dysfunction in equine laminitis. *Equine Vet J,* 43**,** 744-9.
VERVUERT, I., VOIGT, K., HOLLANDS, T., CUDDEFORD, D. & COENEN, M. 2009. Effect of feeding increasing quantities of starch on glycaemic and insulinaemic responses in healthy horses. *Vet J,* 182**,** 67-72.
VETTER, T. R., SCHOBER, P. & MASCHA, E. J. 2018. Diagnostic Testing and Decision-Making: Beauty Is Not Just in the Eye of the Beholder. *Anesth Analg,* 127**,** 1085-1091.
VICK, M. M., ADAMS, A. A., MURPHY, B. A., SESSIONS, D. R., HOROHOV, D. W., COOK, R. F., SHELTON, B. J. & FITZGERALD, B. P. 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. *J Anim Sci,* 85**,** 1144-55.
WAHREN, J., EKBERG, K., JOHANSSON, J., HENRIKSSON, M., PRAMANIK, A., JOHANSSON, B. L., RIGLER, R. & JÖRNVALL, H. 2000. Role of C-peptide in human physiology. *Am J Physiol Endocrinol Metab,* 278**,** E759-68.
WALLER, A. P., BURNS, T. A., MUDGE, M. C., BELKNAP, J. K. & LACOMBE, V. A. 2011. Insulin resistance selectively alters cell-surface glucose transporters but not their total protein expression in equine skeletal muscle. *J Vet Intern Med,* 25**,** 315-21.
WALSH, D. M., MCGOWAN, C. M., MCGOWAN, T., LAMB, S., SCHANBACHER, B. & PLACE, N. J. 2009a. Correlation of Plasma Insulin Concentration with Laminitis Score in a Field Study of Equine Cushing's Disease and Equine Metabolic Syndrome. *J Equine Vet Sci,* 29**,** 87-94.
WALSH, D. M., MCGOWAN, C. M., MCGOWAN, T., LAMB, S. V., SCHANBACHER, B. J. & PLACE, N. J. 2009b. Correlation of Plasma Insulin Concentration with Laminitis Score in a Field Study of Equine Cushing's Disease and Equine Metabolic Syndrome. *J Equine Vet Sci,* 29**,** 87-94.
WARNKEN, T., DELAROCQUE, J., SCHUMACHER, S., HUBER, K. & FEIGE, K. 2018. Retrospective analysis of insulin responses to standard dosed oral glucose tests (OGTs) via naso-gastric tubing towards definition of an objective cut-off value. *Acta Vet Scand,* 60**,** 4.
WARNKEN, T., HUBER, K. & FEIGE, K. 2016. Comparison of three different methods for the quantification of equine insulin. *BMC Vet Res,* 12**,** 196.
WHITEHEAD, J. P., RICHARDS, A. A., HICKMAN, I. J., MACDONALD, G. A. & PRINS, J. B. 2006. Adiponectin--a key adipokine in the metabolic syndrome. *Diabetes Obes Metab,* 8**,** 264-80.
WIEDEMANN, S. J., RACHID, L., ILLIGENS, B., BÖNI-SCHNETZLER, M. & DONATH, M. Y. 2020. Evidence for cephalic phase insulin release in humans: A systematic review and meta-analysis. *Appetite,* 155**,** 104792.
WILCOX, G. 2005. Insulin and insulin resistance. *Clin Biochem Rev,* 26**,** 19-39.
WOOLDRIDGE, A. A., EDWARDS, H. G., PLAISANCE, E. P., APPLEGATE, R., TAYLOR, D. R., TAINTOR, J., ZHONG, Q. & JUDD, R. L. 2012. Evaluation of high-molecular weight adiponectin in horses. *Am J Vet Res,* 73**,** 1230-1240.
WOOLDRIDGE, A. A., WAGUESPACK, R. W., SCHWARTZ, D. D., VENUGOPAL, C. S., EADES, S. C. & BEADLE, R. E. 2014. Vasorelaxation responses to insulin in laminar vessel rings from healthy, lean horses. *Vet J,* 202**,** 83-8.

WYLIE, C. E., COLLINS, S. N., VERHEYEN, K. L. & NEWTON, J. R. 2013. Risk factors for equine laminitis: a case-control study conducted in veterinary-registered horses and ponies in Great Britain between 2009 and 2011. *Vet J,* 198**,** 57-69.

WYLIE, C. E., COLLINS, S. N., VERHEYEN, K. L. & RICHARD NEWTON, J. 2011. Frequency of equine laminitis: a systematic review with quality appraisal of published evidence. *Vet J,* 189**,** 248-56.

WYSE, C. A., MCNIE, K. A., TANNAHILL, V. J., MURRAY, J. K. & LOVE, S. 2008. Prevalence of obesity in riding horses in Scotland. *Vet Rec,* 162**,** 590-1.

YODER, S. M., YANG, Q., KINDEL, T. L. & TSO, P. 2010. Differential responses of the incretin hormones GIP and GLP-1 to increasing doses of dietary carbohydrate but not dietary protein in lean rats. *Am J Physiol Gastrointest Liver Physiol,* 299**,** G476-85.

# General Appendix

## Chapter 5

### Appendix 1:

Age, body weight, body condition score and girth and belly circumference in all (*n* = 12), insulin dysregulated (*n* = 6) and non-insulin dysregulated (*n* = 6) ponies.

### Appendix 2:

Serum insulin concentration before (0 min) and 120 min following an oral glucose test in 12 ponies, separated into insulin dysregulated (ID, *n* = 6) and non-ID (*n* = 6).

### Appendix 3:

Time (min) to consume forages fed as 0.25% BW as dry matter.

## Chapter 7:

### Appendix 4:

Grading scheme for hoof morphology and supraorbital fat

### Appendix 5:

Correlation and linear regression from paired analyses of serum insulin concentration in 39 samples on the Immulite 2000XPi and the Immulite 2000 chemiluminescent analysers.

### Appendix 6:

Questionnaire used for owner interviews.

### Appendix 7:

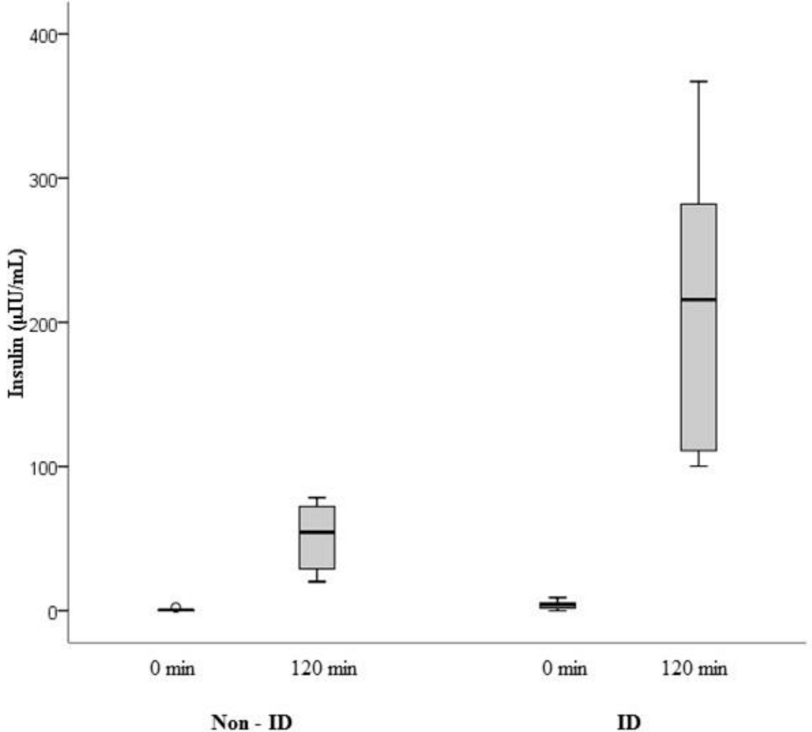
Causal web for EMS. Variables included as potentially causal risk factors are plain text, clinical manifestations are underlined.

# Appendix 1

Age, body weight, body condition score and girth and belly circumference in all (*n* = 12), insulin dysregulated (*n* = 6) and non-insulin dysregulated (*n* = 6) ponies. Data presented as mean ± standard deviation unless otherwise specified.

| | All (*n* = 12) | Insulin dysregulated (*n* = 6) | Non-insulin dysregulated (*n* = 6) |
|---|---|---|---|
| Age (years) | 9.1 ± 3.4 | 10.8 ± 2.6 | 7.3 ± 3.3 |
| Body weight (kg) | 280 ± 49 | 273 ± 15 | 288 ± 73 |
| Girth measurement (cm) | 151 ± 10 | 151 ± 4.8 | 151 ± 13.3 |
| Belly measurement (cm) | 170 ± 14 | 167 ± 6.1 | 173 ± 18.8 |
| Cresty neck score (median, IQR) | 3 (2.6 – 3.9) | 3 (2.88 – 3.63) | 3.25 (2.0 – 4.0) |
| Body condition score (median, IQR, range) | 7.2 (6.0 – 7.7, 4.2-8.5) | 6.9 (5.3 – 7.4, 4.2-7.8) | 7.4 (6.5 – 8.3, 5.8-8.5) |

# Appendix 2




Serum insulin concentration before (0 min) and 120 min following an oral glucose test in 12 ponies, separated into insulin dysregulated (ID, $n$ = 6) and non-ID ($n$ = 6).

# Appendix 3

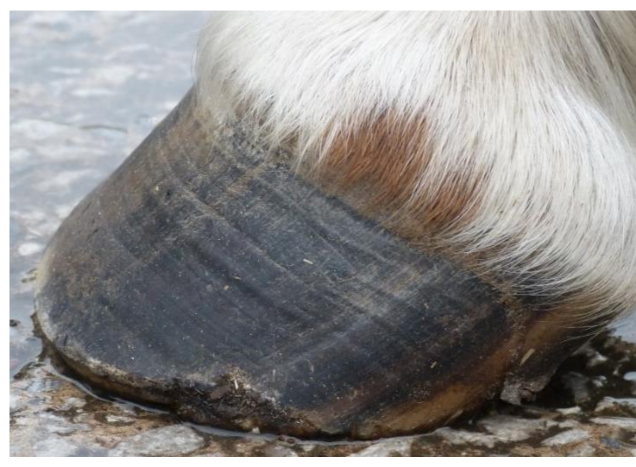
Time (min) to consume forages fed as 0.25% BW as dry matter. Data are expressed as median (interquartile range).

| | All ($n$ = 12) | Insulin dysregulated ($n$ = 6) | Non-insulin dysregulated ($n$ = 6) |
|---|---|---|---|
| Soaked hay | 68.5 (48.3 - 97) | 68.5 (53.8 - 95) | 72 (45.8 – 116.8) |
| Dry hay | 55.5 (49.3 – 76.8) | 55 (42.8 – 70.3) | 57 (50 – 86.8) |
| Haylage | 52.5 (45 – 66.8) | 45 (41.3 – 65.3) | 56.5 (43.8 – 78.8) |
| Glucose/chaff | 22 (15.8 – 37.3) | 19.5 (14.5 – 35.3) | 26.5 (16.8 – 41.3) |

# Appendix 4

Grading Scheme for hoof growth rings and supraorbital fat.

**Hoof growth ring divergence score**

| Grade | Description | Example |
|---|---|---|
| 1 | All growth rings are parallel to the coronary band, or show only very mild separation towards the heel. | |
| 2 | Toward the heel, growth rings show moderate separation but remain straight and/or shown mild distal deviation |  |
| 3 | Towards the heel, growth rings show marked separation and/or marked distal deviation | |

**Hoof growth ring prominence score**

Hooves should be assessed away from any areas of rasping that may have occurred during trimming or farriery

| Grade | Description | Example |
|---|---|---|
| 1 | Hoof capsule is smooth, or growth rings only just visible or palpable | |
| 2 | Moderate depth growth rings clearly visible and palpable | |
| 3 | Prominent, deep growth rings visible | |

**Supraorbital fat score**

Grade 1: The soft tissue within the supraorbital fossa is concave relative to the surrounding bone.

Grade 2: The soft tissue within the supraorbital fossa is level with the surrounding bone.

Grade 3: The soft tissue within the supraorbital fossa is convex relative to the surround bone.

# Appendix 5: Correlation and linear regression from paired analyses of serum insulin concentration in 39 samples on the Immulite 2000XPi and the Immulite 2000 chemiluminescent analysers. Statistical analysis performed using SPSS 24[1].

Equine serum samples (n=39) were analysed on two models of a chemiluminescent analyser (Immulite 2000[2] and Immulite 2000XPi[2]). Descriptive data are shown in Table 1.

Table 1: Descriptive data (median (IQR) for paired analysis of equine serum samples (n=39) on 2 models of a chemiluminescent analyser (Immulite 2000 and Immulite 2000XPi).

| Assay | Median (IQR) |
|---|---|
| Immulite 2000 | 33.45 (12.8 – 74.5) |
| Immulite 2000XPi | 59.50 (23.3 – 126.5) |

Assessment of a scatter plot (Fig. 1) was suggestive of a strong linear relationship between the two variables, with a positively skewed distribution. The distribution of the serum insulin concentration in both data sets was significantly skewed (Shapiro-Wilk test for normality $P$ <0.001, Fig. 2), and Pearson and Spearman's Rank coefficients show a very strong positive correlation of 0.998 and 0.996, respectively ($P$ <0.001).

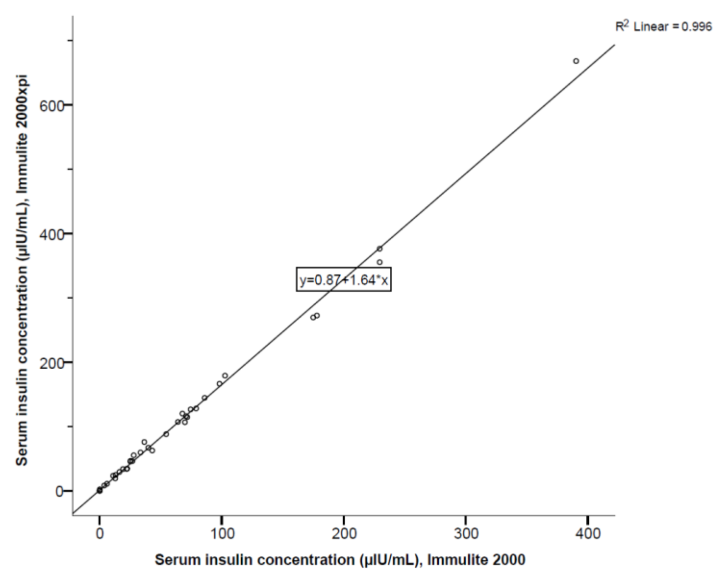



**Figure 1**

Fig. 1 Scatter plot of serum insulin concentration (µIU/mL) with line of best fit from paired analysis with two chemiluminescent analysers (Immulite 2000 (*x*-axis) and Immulite 2000XPi (*y*-axis))

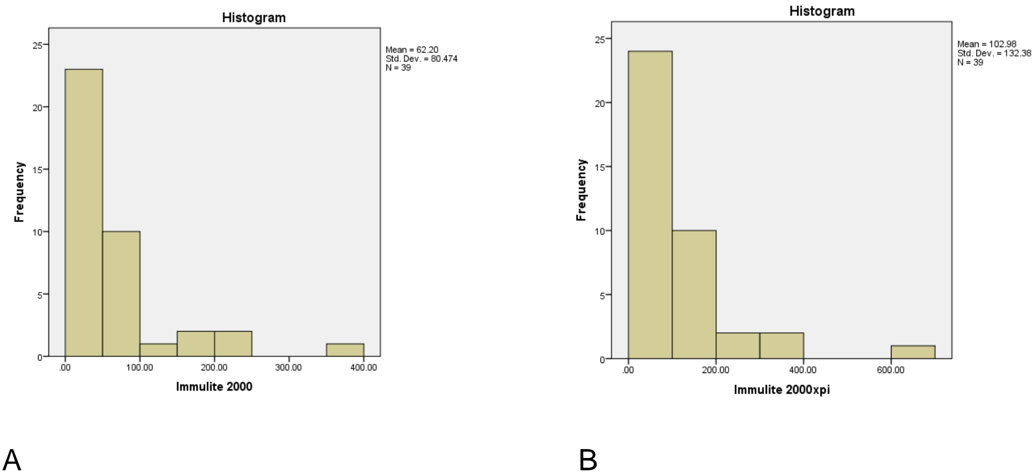



Fig. 2 Histograms showing distribution of serum insulin concentration on two chemiluminscent analysers (Immulite 2000, A and Immulite 2000XPi, B)

### Linear Regression

Simple regression analysis was performed to transform serum insulin concentrations ([insulin]) using the older Immulite 2000 analyser as the independent variable ($[\text{insulin}]_{2000XPi}$) and the newer Immulite 2000XPi as the dependent variable ($[\text{insulin}]_{2000}$). Given the very strong correlation, ($r = 0.998$, $R^2 = 0.996$) linear regression was used. The intercept was 0.87 and gradient (95% CI) 1.64 (1.61 – 1.68), resulting in the regression model:

$$[\text{insulin}]_{2000XPi} = 0.87 + 1.64([\text{insulin}]_{2000}) \qquad (P < 0.001)$$

### Assessment of fit

R squared value was 0.996, so 99.6% of the variation in $[\text{insulin}]_{2000XPi}$ can be explained by the model containing $[\text{insulin}]_{2000}$. Residuals were approximately normally distributed (Fig. 3). A scatter plot of the standardised predicted values versus standardised residuals (Fig. 4) showed heteroscedasticity.

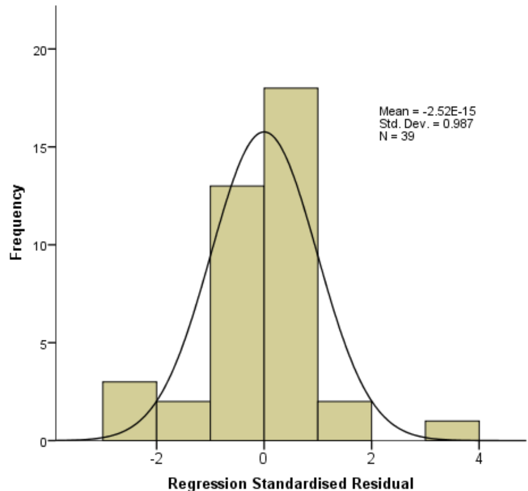



Fig. 3 Histogram of the frequency of residuals of the linear regression model showing an approximately normal distribution

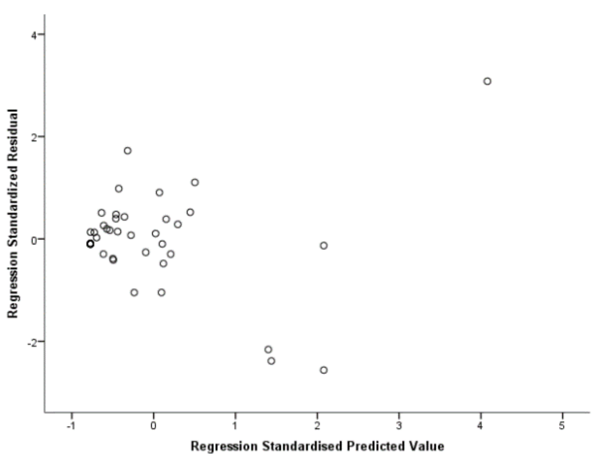



Fig. 4. Scatterplot of residuals against standardised predicted value showing heteroscedasticity.

[1]IBM Corp. New York, USA

[2]Siemens, Healthcare, Camberley, Surrey, UK

# Appendix 6: Questionnaire used for owner interviews

**Prevalence and risk factors for Equine Metabolic syndrome**

**Client Questionnaire interview schedule**

**Name of owner:** ___________________________ **Date**: ____________________________

## A. Signalment/use

1. **Name of pony** ___________________
2. **Breed?** ___________________
3. **Colour?** ___________________
4. **Age?** ___________________ years
5. **Sex?** STALLION / MARE / GELDING
6. **For how many years have you had the pony?** _____________ years
7. **What activities do you do currently with your pony?**
   a. SHOWING / BREEDING / RIDING / DRIVING / COMPANION / PET / OTHER _______
   b. Has your pony had a prior showing/competition career? (YES/NO)
8. **Would you regard your pony as:** VERY THIN / SLIGHTLY UNDERWEIGHT/ IDEAL WEIGHT / SLIGHTLY OVERWEIGHT / VERY OVERWEIGHT?
   a. **Has your pony previously been overweight or very overweight?** (Y / N)
   b. **Is your pony the same weight year round?** (Y / N) (if no _______________________)
9. **Do you monitor your pony's weight?** - weigh tape / weigh bridge? (Y / N)
10. **Do you know the most recent weight of your pony?** __________ On What Date? ________
11. **Is your pony pregnant?** If yes – how many weeks / due date? (exclude third trimester (> 30 weeks) and lactating)
12. **Has your mare had foals in previous years?** 2014 / 2013 / 2012 / 2011 / 2010 Number of foals before 2010? _______

## B. Exercise

1. **How many hours per week does you pony spend in the following activities:-**

Schooling - lunged ☐

Schooling - ridden, flat-work ☐

Schooling - ridden, jumping ☐

Hacking ☐

Driving ☐

Other - ___________________________________

2. **Roughly what percentage of the total exercise time each week does your pony spend:-**

Walking ☐ % of total exercise time per week

Trotting ☐

Cantering ☐

Galloping ☐

Jumping ☐

3. **Overall, what level of work do you consider your pony to be doing?**

☐ light ☐ moderate ☐ intense

## C. Feeding/housing

**1. Total number of horses on the premises** ________

2. **How many hours a day is your pony:-**

Turned out at grass:- ☐ hrs/day **in summer**

Turned out at grass:- ☐ hrs/day **in winter**

Turned out to graze-poor paddock (e.g. sand or wood-chip arena):- ☐ hrs/day **in summer**

Turned out in graze-poor paddock (e.g. sand or wood-chip arena):- ☐ hrs/day **in winter**

Stabled for:- hours per day in summer

Stabled for:- ☐ hours per day in winter

3. **What Bedding do you use?** Straw / Shavings / Rubber matting / Paper / other / N/A

4. **Do you use winter Rugging?** – Y/N – just at turnout / turnout and stable - Type:_________________
5. **Do you use Summer Rugging?** – Y/N just at turnout / turnout and stable - Type:_________________
6. **If turned out to grass, do you ever limit your pony's grazing by use of:-**

| | | |
|---|---|---|
| Strip-grazing? | Yes ☐ | No ☐ |
| Topping? | Yes ☐ | No ☐ |
| Grass muzzle? | Yes ☐ | No ☐ |

If YES: ______________________________________________________________________

7. **Besides grazing, what other food does your pony consume each day & in what quantities?**

***Dry HAY*** ☐Yes ☐No

***Amount?*** __________________ kg / lbs / slices / haynets /other______________ **Type of hay?** mixed grass / meadow / clover/ ryegrass other ____________

***SOAKED HAY*** Yes ☐ No ☐

***Presoaked amount =*** _______________ kg / lbs / slices / haynets /other______________

**How long do you soak the hay?** ________________

**What temperature is the water?** _______________

**Other (e.g. steamed)** ____________________

***Haylage*** Yes ☐ No ☐

***Amount?*** __________________ kg / lbs / slices / haynets /other______________

***Pony nuts/cubes*** Yes ☐ No ☐

***Amount?*** __________________ kg / lbs / scoops / other __________

Trade name of product/s used_____________________________

***Chaff (short chop proprietary feed e.g. HiFi, Alfa A etc.)*** Yes ☐ No ☐

***Amount?*** __________________ kg / lbs / scoops / other____

Trade name of product/s

used…………………………………………………………………………

***Coarse mix / Concentrates*** Yes ☐ No ☐

***Amount?*** __________________ kg / lbs / scoops / other____

Trade name of product/s

used…………………………………………………………………………

***Other Coarse mix / Concentrates*** Yes ☐ No ☐

***Amount?*** __________________ kg / lbs / scoops / other ____

Trade name of product/s

used………………………………………………………………………….

***Balancers?*** Yes ☐ No ☐

***Amount?*** __________________ kg / lbs / scoops / other __________

Trade name of product/s

used…………………………………………………………………………

***Vitamin/Mineral supplements***

| | | |
|---|---|---|
| Joint supplements | NAME __________ | Amount __________ |
| Vitamins/minerals | NAME __________ | Amount __________ |
| Hoof supplements | NAME __________ | Amount __________ |
| Electrolytes | NAME __________ | Amount __________ |
| Pro/Prebiotics | NAME __________ | Amount __________ |
| Herbal supplements | NAME __________ | Amount __________ |
| Other (Please specify) | NAME __________ | Amount __________ |

## D. Management

**1. How do you maintain your pony's feet?**

a. TRIMMING or SHOEING / CORRECTIVE TRIMMING (EXPLAIN) / CORRECTIVE FARRIERY (EXPLAIN) ______________________________

b. IN FRONT +/- BEHIND? How often? __________

**2. How often are your pony's teeth examined/rasped?**

Never / 6 months / 12 months / 24 months / only if there is a problem / never

a. **By who?** - Vet / Lay equine Dentist

## E. Medical history

**1. Has your horse ever suffered from laminitis?** Yes ☐ No ☐

For each episode grade the severity : (**4**:unwilling to walk/lying down; **3**: Quite lame (unwilling to lift up a front leg); **2**: Pottery at walk; **1**: only pottery on corners/Hard surfaces)

| | Episode 1 | Episode 2 | Episode 3 | Episode 4 | Episode 5 |
|---|---|---|---|---|---|
| **Month/Year** | | | | | |
| **Severity** | | | | | |
| **Veterinary advice? (Y/N)** | | | | | |
| **Treatment?** | | | | | |

**Did you make permanent changes to its management after the episode of laminitis?**

Yes ☐ No ☐ ***Yes*** *– when and what were they?*

_________________________________

**2. Have you ever had your pony tested for:**

Equine metabolic syndrome? Date: ______ Result: ________

Equine Cushing's disease? Date:______________ Result:_________________

**Is your pony on any treatment for this?** Yes ☐ No ☐

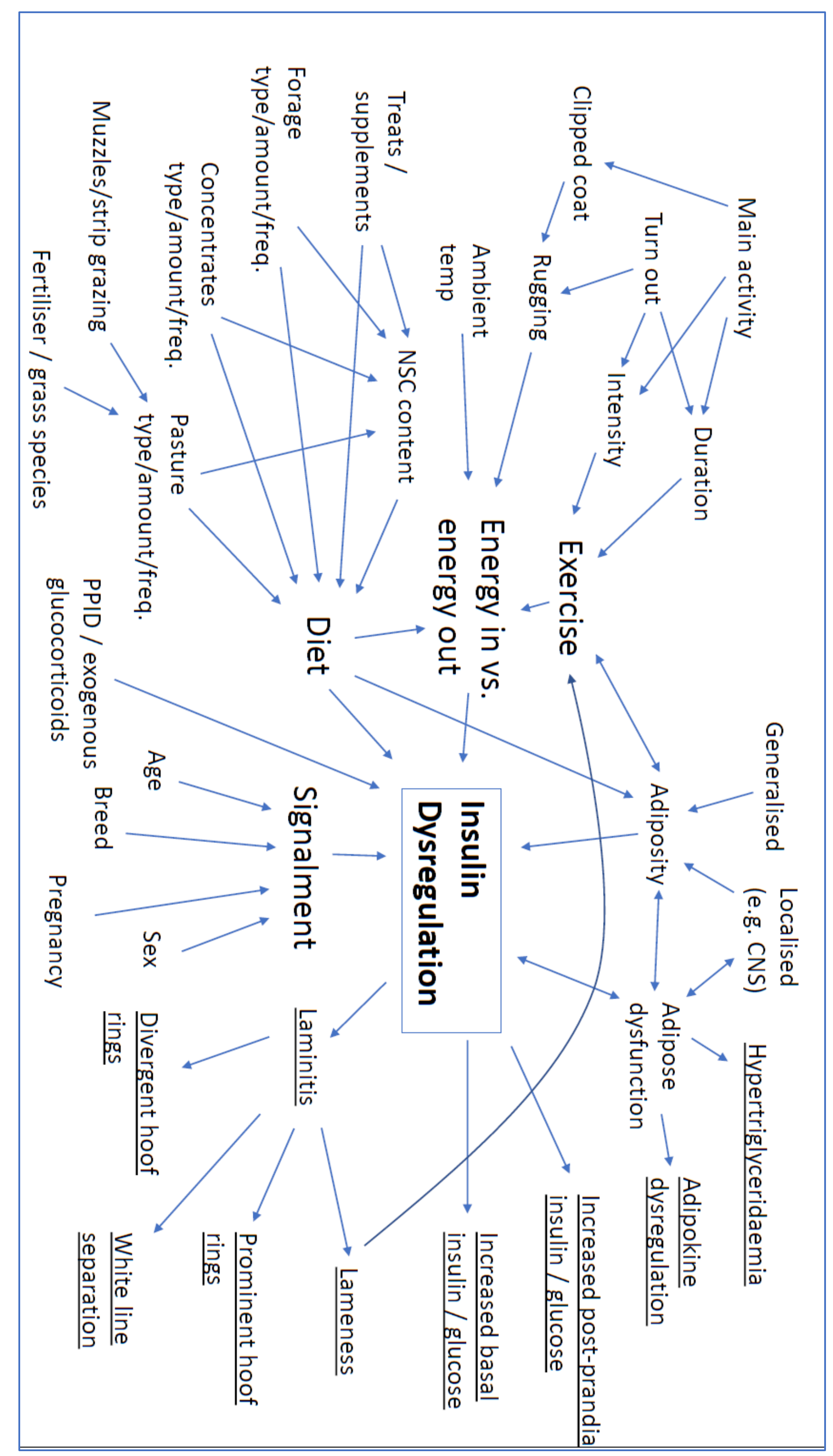
______________

**3. Has your pony ever had any other medical conditions or lameness diagnosed?**

| | Condition 1 | Condition 2 | Condition 3 | Condition 4 |
|---|---|---|---|---|
| **Month/Year** | | | | |
| **Diagnosis** | | | | |

| **Treatment?** | | | | |
|---|---|---|---|---|
| **Outcome?** | | | | |

Appendix 7 Causal web for EMS. Variables included as potentially causal risk factors are plain text, clinical manifestations are underlined.



# Supplementary Information

1. Published version of Chapter 2

CARSLAKE, H., KARIKOSKI, N., PINCHBECK, G. & MCGOWAN, C. 2016. Serum insulin concentration in horses: Effect of storage and handling. *Vet J,* 211**,** 94-6. DOI: 10.1016/j.tvjl.2016.02.016

2. Published version of Chapter 3

CARSLAKE, H. B., PINCHBECK, G. L. & MCGOWAN, C. M. 2017. Evaluation of a Chemiluminescent Immunoassay for Measurement of Equine Insulin. *J Vet Intern Med,* 31**,** 568-574. DOI: 10.1111/jvim.14657

3. Published version of Chapter 5

CARSLAKE, H. B., ARGO, C. M., PINCHBECK, G. L., DUGDALE, A. H. A. & MCGOWAN, C. M. 2018. Insulinaemic and glycaemic responses to three forages in ponies. *Vet J,* 235**,** 83-89. DOI: 10.1016/j.tvjl.2018.03.008

4. Published version of Chapter 7

CARSLAKE, H. B., PINCHBECK, G. L. & MCGOWAN, C. M. 2021. Equine metabolic syndrome in UK native ponies and cobs is highly prevalent with modifiable risk factors. *Equine Vet J,* 53**,** 923-934. DOI: 10.1111/evj.13378