# Insulin Sensitivity and Postprandial Insulin Response in Equines

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2017

Acta Universitatis agriculturae Sueciae
2017:82

Cover: Icelandic horses (pixabay.com)

ISSN 1652-6880
ISBN (print version) 978-91-7760-054-1
ISBN (electronic version) 978-91-7760-055-8

Print: SLU Service/Repro, Uppsala 2017

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## Abstract

A metabolic syndrome in equines was first proposed in 2002, describing a cluster of clinical abnormalities including obesity, insulin resistance (IR), fasting- and postprandial hyperinsulinemia and a predisposition for laminitis. It has been established that intake of grain-based diets rich in non-structural carbohydrates (NSC) contribute to development of IR and hyperinsulinemia. A causal link between hyperinsulinemia and laminitis in horses has also been proven. A correct diagnosis of IR requires the use of complex and expensive tests, which complicates clinical identification of horses at risk for laminitis. The overall aim for this thesis was to study factors that influence insulin sensitivity (IS) in horses and to study the relationship between IS and the postprandial insulin response. In addition, a simple field test for identification of horses with postprandial hyperinsulinemia was evaluated. By feeding horses increasing amounts of a forage-based diet low in NSC, a 9% increase in body weight was obtained, but there was no decrease in the horses' IS. This indicates that the dietary content of NSC has a greater impact on alterations in IS in horses than short-term weight gain. An oral sugar test (OST) was developed and was found to be practical for diagnosis of postprandial hyperinsulinemia in horses under field settings. A hyperbolic relationship was found between OST-derived indices of β-cell response (based on postprandial insulin data) and quantitative measures of IS in horses. This implies that the β-cell response depend on the prevailing IS. Results also confirm that the OST primarily is a test that estimates the β-cell response rather than IS. The postprandial insulin response was evaluated using forage diets differing in NSC content. It was found that the postprandial insulin response depended on both the NSC content in forage and the horse's IS, but the effect of IS was diminished when the forage NSC content was low. This indicates, that horses with IR and an augmented β-cell response should be a fed forage diet with low content of NSC, in order to attenuate the postprandial insulin response and thereby decrease the risk for laminitis. This work adds to the knowledge about the pathophysiology, clinical management and recognition of horses with IR and hyperinsulinemia.



*Keywords:* Beta-cell response, equine metabolic syndrome, forage, hyperbolic relationship, hyperinsulinemia, insulin dysregulation, insulin resistance, non-structural carbohydrates, obesity, oral sugar test, water soluble carbohydrates.



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# Dedication

To my family and friends

*Felix qui potuit rerum cognoscere causas*
*”Lycklig den som inser sakers orsaker”*
Vergilius, Georgica

# Contents

# List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Lindåse, S.*, Nostell, K., Müller, C., Jensen-Waern, M. & Bröjer, J. (2016). Effects of diet-induced weight gain and turnout to pasture on insulin sensitivity in moderately insulin resistant horses. *American Journal of Veterinary Research* 77(3), 300-309.

II Lindåse, S.*, Nostell, K. & Bröjer, J. (2016). A modified oral sugar test for evaluation of insulin and glucose dynamics in horses. *Acta Veterinaria Scandinavica* 58(suppl 1):64.

III Lindåse, S.*, Nostell, K., Söder, J. & Bröjer, J. (2017). Relationship between β-cell response and insulin sensitivity in horses based on the oral sugar test and the euglycemic hyperinsulinemic clamp. *Journal of Veterinary Internal Medicine* 31(5), 1541-1550.

IV Lindåse, S.*, Nostell, K., Müller, C. & Bröjer, J. Evaluation of glucose and insulin response to haylage diets with different content of water soluble carbohydrates in two horse breeds. Submitted manuscript.

Papers I-III are reproduced with the permission of the publishers.

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## Related publications not included in the thesis

- Bröjer, J., Lindåse, 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. *Veterinary Internal Medicine* 27(6), 1543–1550.

- Nostell, K., Lindåse, S. & Bröjer, J. (2016). Blood pressure in Warmblood horses before and during a euglycemic-hyperinsulinemic clamp. *Acta Veterinaria Scandinavica* 58(suppl 1):65.

# Abbreviations

| | |
|---|---|
| AIRg | Acute insulin response to glucose |
| $AUC_{GLU}$ | Total area under the curve for glucose |
| $AUC_{GLU\text{-}60}$ | Area under the curve for glucose at 60 min |
| $AUC_{INS}$ | Total area under the curve for insulin |
| $AUC_{INS\text{-}60}$ | Area under the curve for insulin at 60 min |
| $AUC_{INS\text{-}180}$ | Area under the curve for insulin at 180 min |
| $AUC_{INS/GLU\text{-}30}$ | Area under the curve for insulin/glucose at 30 min |
| $AUC_{INS/GLU\text{-}120}$ | Area under the curve for insulin/glucose at 120 min |
| $AUC_{INS/GLU\text{-}180}$ | Area under the curve for insulin/glucose at 180 min |
| BCS | Body condition score |
| BW | Body weight |
| CGIT | Combined glucose-insulin tolerance test |
| CNS | Cresty neck score |
| CV | Coefficient of variation |
| DI | Disposition index |
| DM | Dry matter |
| EHC | Euglycemic hyperinsulinemic clamp |
| EMS | Equine metabolic syndrome |
| FSIGTT | Frequently sampled intravenous glucose tolerance test |
| GLP-1 | Glucagon-like peptide 1 |
| HW | High water soluble carbohydrate content |
| ICC | Intraclass correlation coefficient |
| ID | Insulin dysregulation |
| $INS_{INDEX}$ | Insulinogenic-index |
| IR | Insulin resistance |
| IS | Insulin sensitivity |
| $ISI_{60}$ | 60 min insulin sensitivity index |
| $ISI_{90}$ | 90 min insulin sensitivity index |
| $ISI_{COMP}$ | Composite whole-body insulin sensitivity index |

| | |
|---|---|
| $ISI_{PEAK}$ | Peak insulin sensitivity index |
| LW | Low water soluble carbohydrate content |
| M | Mean glucose disposal rate |
| M/I | Mean glucose disposal rate per unit of insulin |
| MCR | Metabolic clearances rate for insulin |
| ME | Metabolizable energy |
| MW | Medium water soluble carbohydrate content |
| NIMGU | Non-insulin-mediated glucose uptake |
| NIR | Normal insulin regulation |
| NSC | Non-structural carbohydrates |
| OGTT | Oral glucose tolerance test |
| OST | Oral sugar test |
| $Peak_{GLU}$ | Peak concentration for glucose |
| $Peak_{INS}$ | Peak concentration for insulin |
| RC | Repeatability coefficient |
| SD | Standard deviation |
| Sg | Glucose effectiveness |
| Si | Insulin sensitivity index |
| $T\text{-}peak_{INS}$ | Time (min) to peak concentration for insulin |
| WSC | Water soluble carbohydrates |
| WSC-f | Water soluble carbohydrates - fructans |

# 1 Introduction

## 1.1 Laminitis

Laminitis is a painful condition in equines in which there is a failure of the laminar tissue between the inner hoof wall and the pedal bone (Obel, 1948; Pollitt, 1996). Laminitis can result in permanent lameness in horses and has major economic and welfare implications. In a survey performed by the United states Department of Agriculture in 2000, it was found that 13% of horse farmers had at least one case of laminitis during the previous year and 5% of these horses died or were euthanized. There are no published results on the prevalence of laminitis in horses in Sweden. However, by extrapolation of insurance data based on an approximated horse population of 360,000 individuals, it was estimated that 15 horses per day develop laminitis and that two horses per day are euthanized due to laminitis[1].

There are several pathological conditions implicated in the development of laminitis including grain overload, mechanical overload, systemic inflammatory responses (colitis, enteritis and acute metritis) and endocrine disorders (insulin resistance and pituitary pars intermedia dysfunction) (Bailey *et al.*, 2004; Geor, 2013b). Insulin resistance (IR) and hyperinsulinemia (elevated insulin concentrations in blood) are frequently associated with laminitis in horses and ponies (Treiber *et al.*, 2006a; Treiber *et al.*, 2006b; Bailey *et al.*, 2008; Geor, 2008; Carter *et al.*, 2009c). A causal link between hyperinsulinemia and laminitis has also been proven, by induction of laminitis in healthy horses and ponies by experimentally induced continuous hyperinsulinemia (Asplin *et al.*, 2007; De Laat *et al.*, 2010). Laminitis often occur during pasture periods when horses have unlimited access to grass rich in non-structural carbohydrates

1. Personal Communication, Dr. Ove Wattle, Senior Lecturer at the Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden.

(NSC), giving rise to the definition "pasture associated laminitis" (Treiber *et al.*, 2006b; Carter *et al.*, 2009c).

## 1.2 Insulin sensitivity and insulin resistance

Insulin is a peptide-hormone synthesized and secreted by β-cells in the islets of Langerhans in response to elevated plasma glucose concentrations. The main metabolic action of insulin is to maintain whole-body glucose homeostasis, but insulin also exercises regulatory effects on lipid and protein metabolism as well as on vascular endothelial function (Wilcox, 2005). Insulin sensitivity (IS) is defined as the ability of insulin to enhance glucose utilization by promoting glucose uptake into skeletal muscle and adipose tissue and by increasing hepatic glycogen storage and reducing hepatic glucose production. Insulin resistance defines a state in which physiological concentrations of insulin fail to induce a normal response in the target tissues (Kahn, 1978; Bergman *et al.*, 1985; Trout *et al.*, 2007). To maintain euglycemia, the β-cells compensate for IR by increasing the secretion of insulin into the blood stream resulting in hyperinsulinemia (Wilcox, 2005). Hyperinsulinemia can also develop as a consequence of decreased hepatic insulin clearance, as part of the compensatory response to IR (Mittelman *et al.*, 2000; Erdmann *et al.*, 2008; Toth *et al.*, 2010; Ader *et al.*, 2014).

In humans, this compensated state of IR almost inevitably progresses towards decompensated IR, resulting in impaired glucose tolerance and type II diabetes mellitus (Wallace & Matthews, 2002; Wilcox, 2005). In horses, type II diabetes mellitus is rare and the β-cells' appear to fully compensate for the IR over a long time period, i.e., the horse remains in a state of compensated IR. Although the most accepted theory is that hyperinsulinemia, as part of the β-cells' compensatory response, is related to the prevailing IR (Bergman *et al.*, 2002; Hoffman *et al.*, 2003; Wilcox, 2005; Toth *et al.*, 2010), it has been suggested that hyperinsulinemia is the primary requisite and IR the compensatory or adaptive response (Shanik *et al.*, 2008; Corkey, 2012b; Corkey, 2012a). In horses, it has recently been proposed that postprandial hyperinsulinemia might occur without an adaptive decrease in IS (Frank & Tadros, 2014).

## 1.3 Equine metabolic syndrome

A metabolic syndrome in equines, the "equine metabolic syndrome" (EMS) was first proposed in 2002 and refers to a cluster of clinical abnormalities predisposing horses and ponies for laminitis (Johnson, 2002). The characteristics and the name of the syndrome were adopted from the human metabolic

syndrome, which is a collection of risk factors including central (visceral) obesity, elevated blood pressure, IR, hyperinsulinemia and dyslipidemia associated with type II diabetes mellitus and cardiovascular disease (Fulop *et al.*, 2006). In 2010, a consensus statement on EMS was published in which general obesity and/or regional adiposity, IR, fasting hyperinsulinemia, abnormal insulin responses to oral or intravenous glucose administration and a predisposition for laminitis were described as the key components. Dyslipidemia, hypertension and increased systemic inflammation were also discussed as potential additional components of the syndrome (Frank *et al.*, 2010). Through the years, the definition of which metabolic characteristics that should be included in the EMS phenotype have varied and it is still debated which metabolic traits that are important for the development of laminitis. Lately, the role of postprandial hyperinsulinemia has been in focus. A new term, insulin dysregulation (ID), was proposed in 2014, based on the hypothesis that postprandial hyperinsulinemia may occur in horses without concurrent IR (Frank & Tadros, 2014). Insulin dysregulation has since then been used to describe horses expressing any combination of fasting hyperinsulinemia, elevated insulin response to oral or intravenous glucose administration or IR.

Anecdotally, certain breeds of ponies and horses (e.g. New Forest, Shetland and Welsh Mountain ponies as well as Icelandic, Morgan and Andalusian horses) more commonly exhibit the EMS phenotype compared to light-bred horses (e.g. Thoroughbreds and Standardbreds). These EMS predisposed horses are often referred to as "easy-keepers", indicating that they require a lower intake of energy compared to normal or "hard-keepers" to maintain their body condition. An inherited predisposition for EMS has been proposed, which is based on the assumption that ponies and certain breeds of horses genetically have adapted to survival under conditions with low or limited feed availability (Treiber *et al.*, 2006b; McCue *et al.*, 2015). In support of this, several studies have found differences in IS and postprandial insulin responses between healthy horses of different breeds (Bröjer *et al.*, 2013; Bamford *et al.*, 2014; Manfredi, 2016; Jacob *et al.*, 2017).

*Figure 1.* Shetland pony (left), Icelandic horse (middle) and Standardbred (right). Two EMS predisposed breeds (Shetland pony and Icelandic horse) and one breed (Standardbred) that does not commonly exhibit the EMS phenotype. Photos: S. Lindåse., K. Nostell.

## 1.4 Effects of obesity on insulin sensitivity

Obesity is a pathological condition that anecdotally has been described as a growing problem in horses worldwide. Between 45 and 51% of horses have been reported to be overweight or obese in different population studies, which confirms a high prevalence of obesity in the equine population (Wyse *et al.*, 2008; Pratt-Phillips *et al.*, 2010; Thatcher *et al.*, 2012). The most common method for assessment of general obesity in horses is body condition scoring (BCS), using a scale between 1 and 9 (Henneke *et al.*, 1983). This subjective scoring system was originally developed for Quarter horses and is therefore not adjusted for use in other breeds or in ponies. Currently, there is no clear definition of what is considered as obesity in horses and ponies. Different studies have used different cut-off limits on the 9-point BCS scale to define general obesity, starting at grade 6 and above (Treiber *et al.*, 2006b; Vick *et al.*, 2006; Bailey *et al.*, 2008; Carter *et al.*, 2009a; Thatcher *et al.*, 2012).

Obesity has been considered a key feature of EMS (Johnson, 2002) and an important risk factor for development of laminitis (Treiber *et al.*, 2006b; Carter *et al.*, 2009c). In a study of 107 horses and ponies with pasture associated laminitis, treated at a first opinion hospital in the UK, it was found that 83% were overweight or obese (Menzies-Gow *et al.*, 2010). Several studies have also found an association between obesity and IR/hyperinsulinemia in horses and ponies (Hoffman *et al.*, 2003; Frank *et al.*, 2006; Vick *et al.*, 2006). However, hyperinsulinemia has also been detected in lean ponies (Bailey *et al.*, 2008; Borer *et al.*, 2012) and obesity has been observed in horses and ponies with normal IS (Carter *et al.*, 2009a; Ungru *et al.*, 2012). This implies that obesity is not always related to hyperinsulinemia and IR in horses.

The potential effect of obesity eliciting IR has been explored in weight gain and weight loss studies of horses and ponies. A 20% weight gain in Arabian geldings was associated with decreased IS (Carter *et al.*, 2009b), whereas a 15% weight gain in Thoroughbred geldings did not alter IS (Quinn *et al.*, 2008). A 7% (McGowan *et al.*, 2013) or 14% (Ungru *et al.*, 2012) reduction in body weight (BW) improved IS in obese and IR ponies and horses. However, ponies and horses in theses weight loss studies were also subjected to a low intensity exercise program, which might have influenced IS.

Increased regional adiposity, especially when deposited along the crest of the neck of horses and ponies has also been suggested to be associated with IR and laminitis (Johnson, 2002; Frank *et al.*, 2006; Treiber *et al.*, 2006b). During 2009, a cresty neck scoring system (CNS) was developed for assessment of local fat deposition on the neck of horses and ponies (Carter *et al.*, 2009a). The CNS has a scale of 0 to 5, where a score of $\geq 3$ is defined as a “cresty neck”, i.e., an abnormal fat accumulation in the neck area.

## 1.5 Effects of diet on insulin sensitivity and postprandial insulin response

Dietary management provides the core in the control and prevention of IR, hyperinsulinemia and laminitis in horses and ponies. Several studies in horses have shown an association between adaptation to diets rich in NSC and postprandial hyperinsulinemia (Williams *et al.*, 2001; Pratt *et al.*, 2006; Vervuert *et al.*, 2009; Suagee *et al.*, 2013; Pratt-Phillips *et al.*, 2014) and decreased IS (Hoffman *et al.*, 2003; Treiber *et al.*, 2005a; Pratt *et al.*, 2006). Postprandial hyperinsulinemia is often observed in ponies with a previous history of laminitis during pasture periods when the NSC content of grass is high (Hess *et al.*, 2005; Treiber *et al.*, 2006b; Bailey *et al.*, 2008; Treiber *et al.*, 2008). The major problem with the grazing period is that feed intake is not restricted and feed is consumed on an almost continuous basis (McIntosh, 2007).

The frequent occurrence of clinical cases of laminitis during pasture has been attributed to the effect of unlimited intake of grass rich in NSC, causing further deterioration in IR and postprandial hyperinsulinemia in horses with a chronic state of IR (Hess *et al.*, 2005; Treiber *et al.*, 2006b; Treiber *et al.*, 2008; Carter *et al.*, 2009c). One previous study has shown that ponies with recurrent episodes of laminitis were hyperinsulinemic and IR during summer but not during winter compared to control ponies (Bailey *et al.*, 2008). Both groups of ponies had normal BCS, which supports the proposed effect of NSC rich diets on alterations in IS in the absence of obesity.

There is mounting evidence supporting the avoidance of grain-based concentrate feeds and unlimited access to pasture grass in the management and prevention of laminitis in horses and ponies. However, the available information about the effect of NSC content in forage on postprandial insulin response in horses is limited. This is of interest as NSC content in forage may vary widely (Virkajärvi *et al.*, 2012) and as forage constitutes the major part of the ration, a large proportion of daily NSC intake may originate from the forage. Based on clinical experience, a forage diet with < 10 – 12% NSC of dry matter (DM) is currently recommended for horses and ponies with IR and recurrent episodes of laminitis (Geor, 2013a). The use of this arbitrary cut-off value is supported by results from one previous published study (Borgia *et al.*, 2011) in which healthy quarter horses and horses with polysaccharide storage myopathy were fed hay diets with different NSC content. Results showed that the five healthy horses had a minimal insulin response to hay containing 4 and 11% NSC of DM compared to a moderate insulin response to hay containing 17% NSC of DM.

## 1.6 Assessment of insulin sensitivity and postprandial insulin response

Horses rarely progress from compensated to decompensated IR (i.e. β-cell dysfunction) and glucose homeostasis is maintained by an adequate insulin secretion from the β-cells. Measurements of basal plasma glucose concentrations is therefore an unreliable method to detect IR in horses (Treiber *et al.*, 2005b). Fasting hyperinsulinemia can be observed in horses with IR (Frank *et al.*, 2006) and measurement of basal plasma insulin concentrations is a simple method to use in clinical practice. However, factors such as diet, stress and pain might influence the basal secretion of insulin (Frank *et al.*, 2010). Only a weak to moderate negative correlation between basal insulin concentrations and IS quantified using the frequently sampled intravenous glucose tolerance test (FSIGTT; r = -0.52) and the EHC (r = -0.32) has been found in previous studies (Treiber *et al.*, 2005b; Vick *et al.*, 2007). Decreased IS in response to a diet rich in NSC has also been observed in horses without a concurrent increase in basal insulin concentration (Pratt *et al.*, 2006). Furthermore, basal serum insulin concentrations, using the arbitrary cut-off value > 20 μIU/mL for diagnosis of IR (Frank, 2011), did not differ between horses defined as IS and IR using the FSIGTT (Dunbar *et al.*, 2016).

Only quantitative tests such as the EHC and the FSIGTT can accurately measure IR. As glucose is administered intravenously, this avoids confounding stimulatory effects on insulin secretion by incretin hormones secreted in response to a meal (Monzillo & Hamdy, 2003). However, as these tests are time consuming, labor-intensive and expensive to perform, they are impractical to use in clinical settings. The combined glucose-insulin tolerance test (CGIT) (Eiler *et al.*, 2005) is a dynamic non-quantitative intravenous test recommended for diagnosis of IR in a clinical setting (Frank *et al.*, 2010). This test requires placement of intravenous catheters followed by repeated blood sampling for 150 min and is thus a test that is limited to use in hospital settings.

Dynamic tests that involve oral administration of glucose more closely mimic a physiological situation of grazing pasture compared to the intravenous tests. The insulin secretion pattern after oral glucose administration reflects the combined response to several physiological factors such as gastric emptying, rate of intestinal glucose absorption, degree of hepatic glucose trapping and incretin effects (Pacini & Mari, 2003; Kronfeld *et al.*, 2005). As a result, these oral glucose tests do not per se measure IS but rather estimates the β-cell response. In horses, an oral glucose tolerance test (OGTT) (Pratt-Phillips *et al.*, 2015; Smith *et al.*, 2016) or an oral sugar test (OST) (Schuver *et al.*, 2014) can be used to measure the postprandial insulin response and estimates of the β-cell response can be calculated.

### 1.6.1 Euglycemic hyperinsulinemic clamp

The EHC provides a measure of the peripheral IS during steady-state conditions and is the gold standard for diagnosis of IR in humans (DeFronzo *et al.*, 1979; Muniyappa *et al.*, 2008). The method has also been adapted for use in horses (Powell *et al.*, 2002; Annandale *et al.*, 2004; Pratt *et al.*, 2005). The EHC procedure involves a constant rate infusion of insulin resulting in a steady-state level of hyperinsulinemia. At the same time, blood glucose is held constant within the normal range (i.e. around 5 mmol/L) by infusion of glucose at a variable rate. The glucose infusion rate is adjusted based on plasma glucose concentrations that are analyzed at regular intervals (5 to 10 min) throughout the EHC (DeFronzo *et al.*, 1979; Wallace & Matthews, 2002). The procedure usually takes 180 min and the final 60 min are considered to represent steady-state conditions (Pratt *et al.*, 2005). During steady-state conditions of hyperinsulinemia and euglycemia it is assumed that endogenous hepatic glucose production is completely suppressed. Under these circumstances, the quantity of the exogenous glucose infusion rate equals the amount of glucose utilized in the peripheral tissue (i.e. the glucose disposal rate, M-value). Thus, a higher exogenous glucose infusion rate is required to maintain euglycemia during an EHC in individuals with high compared to low IS.

To adjust for small variations in the steady-state glucose concentration during an EHC, a correction factor referred to as the space correction is incorporated in the calculation of the M-value (DeFronzo *et al.*, 1979). When M is related to the steady-state insulin concentration (M/I), this provides a measure of IS in the peripheral tissue. The metabolic clearance rate for insulin (MCR) can be calculated as the constant rate infusion of insulin divided by the increase in plasma insulin concentration above the basal concentration during steady-state (DeFronzo *et al.*, 1979; Monzillo & Hamdy, 2003). This measure provides interesting information as decreased hepatic insulin clearance arises as a compensatory mechanism along with decreased IS (Toth *et al.*, 2010; Ader *et al.*, 2014).

The rate of insulin infused during the EHC determines the level of hyperinsulinemia. Different insulin infusion rates have been used among studies of horses with 3 mIU/kg/min being most commonly used (Annandale *et al.*, 2004; Firshman *et al.*, 2005; Pratt *et al.*, 2005; Pratt *et al.*, 2006; Tiley *et al.*, 2008; Banse & McFarlane, 2014; Pratt-Phillips *et al.*, 2015). It is important to emphasize that comparisons of EHC-derived IS measures among individual horses and between studies are only valid as long as the same infusion rate of insulin is used (Urschel *et al.*, 2013).

### 1.6.2 Frequently sampled intravenous glucose tolerance test

The minimal model analysis of an FSIGTT was first developed in dogs by Bergman and associates in 1979. The test involves intravenous administration of a bolus dose of glucose followed by repeated blood sampling (Bergman *et al.*, 1979). The minimal model of glucose and insulin dynamics relies on the glucose disappearance rate in response to insulin. An adequate insulin secretion is thus a prerequisite, which limits the reliability of the test when performed in patients with reduced β-cell function (Wallace & Matthews, 2002). To increase the precision of the IS estimates in human subjects, modified variants of the FSIGTT were developed including administration of either Tolbutamide (to stimulate endogenous insulin secretion) or exogenous insulin 20 min after the intravenous glucose bolus (Beard *et al.*, 1986; Yang *et al.*, 1987; Finegood *et al.*, 1990). Tolbutamide can only be applied to patients with preserved β-cell function (Wallace & Matthews, 2002). In contrast, the insulin-modified FSIGTT can be used to ensure adequate insulin concentrations during the testing procedure in patients with β-cell dysfunction and type II diabetes mellitus (Quon *et al.*, 1994; Muniyappa *et al.*, 2008).

Hoffman and colleagues were the first to use the insulin-modified FSIGTT for assessment of IS in horses (Hoffman *et al.*, 2003). Since then, the majority of studies performed in horses have used a glucose dose of 300 mg/kg BW and an insulin dose of 20 mIU/kg BW for the insulin-modified FSIGTT (Carter *et al.*, 2009b; Carter *et al.*, 2010; George *et al.*, 2011; Tinworth *et al.*, 2011b; Bamford *et al.*, 2014; Bamford *et al.*, 2016b; Jacob *et al.*, 2017). Plasma glucose and plasma insulin data derived from blood sampling performed during the FSIGTT are then mathematically interpreted using the minimal model analysis (MinMod Millennium software, version 6.02; University of Pennsylvania, Kennett Square, PA, USA). The following parameters are derived: Insulin sensitivity index (Si), glucose effectiveness (Sg), acute insulin response to glucose (AIRg) and disposition index (DI). Si is a measure of the capacity of insulin to promote glucose disposal and inhibit hepatic glucose production and is calculated as the change in glucose per unit change in insulin. Sg estimates the capacity of glucose to mediate its own disposal and inhibit hepatic glucose production when insulin is at a basal level. The AIRg quantifies the endogenous insulin secretion in response to the glucose dose during the first 10 min of the FSIGTT and provides an estimate of the β-cell response. The DI is a measure of the pancreas functionality and is calculated as Si • AIRg (Bergman *et al.*, 2002; Hoffman *et al.*, 2003; Monzillo & Hamdy, 2003).

A moderate to high (r = 0.70 to 0.91) correlation between IS measures derived from the EHC and the FSIGTT (standard or insulin-modified) has been reported in several species including cats, dogs, humans and horses (Finegood

*et al.*, 1984; Saad *et al.*, 1997; Petrus *et al.*, 1998; Pratt-Phillips *et al.*, 2015). Advantages with the minimal modelling of FSIGTT compared to the EHC include the ability to estimate insulin-dependent glucose utilization (Si), insulin-independent glucose utilization (Sg) as well as the β-cell responsiveness to glucose (AIRg) using one single method. The FSIGTT is also technically simpler to perform compared to the EHC. However, as the mathematical models are based on several assumptions about glucose and insulin dynamics, this can result in systematic errors that affect interpretation of test results (Monzillo & Hamdy, 2003; Muniyappa *et al.*, 2008).

For example, the estimates derived from the minimal modelling are defined as steady-state indices but are calculated during dynamic conditions. In addition, the model is unable to separate the effects of insulin to inhibit hepatic glucose production and to promote peripheral glucose uptake. Thus, the estimate for IS (Si) provides an aggregated measure of hepatic and peripheral IS. Since the relative contribution of hepatic glucose production to Si varies with IS, estimates of Si are less reliable for IR individuals compared to IS individuals (Muniyappa *et al.*, 2008). Moreover, a higher mean coefficient of variation (CV; 23.7 vs 14.1%) has been reported for Si derived from an FSIGTT compared to M/I derived from an EHC for repeated tests performed in horses with normal IS (Pratt *et al.*, 2005).

### 1.6.3 Dynamic oral glucose tests

The OGTT is a simple test commonly used for diagnosis of diabetes mellitus in humans (American Diabetes Association, 2014). The original OGTT procedure for horses was developed during the early 1970s as a test for determination of small intestinal malabsorption and pancreatic β-cell dysfunction (Roberts & Hill, 1973). The standard OGTT involves administration of 1 g glucose/kg BW via a nasogastric tube (Pratt-Phillips *et al.*, 2015). Blood samples are obtained at regular intervals (commonly every 30 min) for 4 – 6 hours post administration of glucose. The in-feed OGTT is a variant of the test in which glucose at a dose of 0.75 – 1.5 g is mixed with a small amount of a highly palatable concentrate feed (Bamford *et al.*, 2014; Smith *et al.*, 2016; De Laat & Sillence, 2017).

Administration of glucose via nasogastric tubing during a standard OGTT requires trained personnel and the procedure can potentially induce stress in the horse. The in-feed OGTT is easier to perform during field settings. However, the amount and type of concentrate used in the feed ration can affect the rate of gastric empting and intestinal glucose absorption, complicating comparison of results between studies. Furthermore, feed refusal has been observed in horses using a dose of 1 g glucose/kg BW (De Laat & Sillence, 2017).

The OST was developed and introduced for use in horses in 2010 in the USA (Schuver *et al.*, 2010; Schuver *et al.*, 2014). This test uses commercially available sugar syrup, Karo® light corn syrup, which is composed of different unspecified carbohydrates. The Karo® light corn syrup is syringed orally at a dose of 0.15 ml/kg BW and blood sampling is performed at regular intervals (commonly every 15 to 30 min) for 2 – 3 hours post syrup administration.

A study comparing the in-feed OGTT with the OST in a group of 13 horses and ponies found an 85% agreement in test interpretation between tests when a binary outcome was used (healthy vs ID) (Smith *et al.*, 2016). It is, however, not possible to determine which of the tests (in-feed OGTT or OST) that most correctly identifies ID, as IS was not quantified in these ponies and horses. The main advantage with the OST is that it is easy to perform, which makes it an appropriate test to use under field conditions. Administration of sugar syrup can be performed by the horse owner, which significantly reduces the time spent on sampling and thus the costs for the test. Unfortunately, Karo® light corn syrup is not available in Scandinavian grocery stores, preventing the use of this test in Scandinavian countries.

A standardized meal tolerance test can be used to evaluate the postprandial insulin response in human subjects with diabetes mellitus (Hovorka *et al.*, 1998; Muniyappa *et al.*, 2008; Besser *et al.*, 2013). In horses, there is no available standardized meal tolerance test protocol, but the insulin response to different meals (forage-based, grain-based etc.) has been investigated in different studies (Williams *et al.*, 2001; Borgia *et al.*, 2011; Jacob *et al.*, 2017). The advantage of evaluating the insulin response to a meal compared to an oral bolus of glucose or glucose syrup is that it reflects a more physiologic digestive process and response to dietary sugars.

### 1.6.4 Insulin sensitivity indices from oral glucose tests

Even though the dynamic OGTT and OST does not measure IS per se, different indices can be calculated based on data from these tests in order to estimate IS. Indices such as the composite whole-body insulin sensitivity index ($ISI_{COMP}$) (Matsuda & DeFronzo, 1999), Gutt index (Gutt *et al.*, 2000), Stumvoll index (Stumvoll *et al.*, 2000) and Belfiore index (Belfiore *et al.*, 1998) have been used in different studies in humans. A moderate to high linear correlation ($r = 0.63$ to $0.73$) between the most commonly used index in humans, $ISI_{COMP}$ (Matsuda & DeFronzo, 1999), and quantitative measurements of IS obtained by the EHC has been shown in humans and in horses (Matsuda & DeFronzo, 1999; Pratt-Phillips *et al.*, 2015).

## 1.7 Non-linear inverse relationship between insulin sensitivity and β-cell response

In humans, a non-linear inverse relationship between β-cell response and IS was first described in the early 1980s (Bergman *et al.*, 1981). This finding implies that the β-cell response and IS are dependent on each other, where changes in one feature is mirrored by a reciprocal adaptation in the other (Kahn, 2003). Due to the nature of this non-linear inverse relationship, the magnitude in β-cell response that follows a change in IS will depend on the prevailing IS. This means that individuals with high IS will have small increases in their β-cell response following large decreases in IS, whereas individuals with markedly decreased IS will have large changes in their β-cell response along with small changes in IS (Figure 2).

A non-linear inverse relationship between the peak insulin concentration (an estimate of β-cell response) derived from an in-feed OGTT and IS measures from an FSIGTT has previously been reported in horses (Bamford *et al.*, 2014).

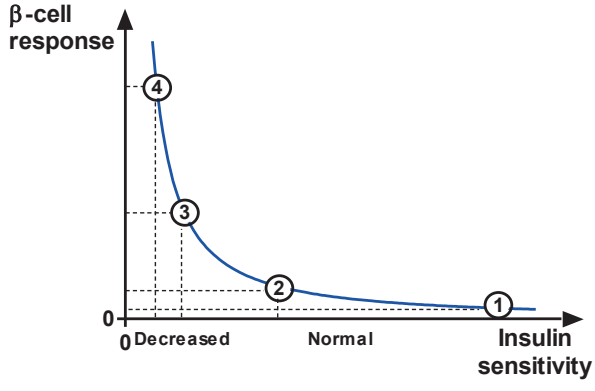


*Figure 2.* Schematic diagram illustrating the characteristics of a non-linear inverse relationship between insulin sensitivity (IS) and β-cell response. State 1: An individual with high IS and normal β-cell response. State 2: The same individual after a large decrease in IS resulting in a small increase in β-cell response. State 3: An individual with low IS. State 4: The same individual after a small decrease in IS resulting in a large increase in β-cell response.

Under specific circumstances, this non-linear inverse relationship between the β-cell response and IS can be described by a rectangular hyperbolic function ($y = \text{constant} \cdot 1/x$, or $y \cdot x = \text{constant}$) (Bergman *et al.*, 1981; Kahn *et al.*, 1993). The rectangular hyperbolic function can be re-expressed as a linear model by log-transformation of data producing the following equation: $\text{Ln}(y) = \text{constant} + \beta \cdot \text{Ln}(x)$, where β is the regression coefficient. The β-cell response is defined as the dependent variable (y) whereas IS is defined as the independent

variable (x). The criterion for a rectangular hyperbolic relationship is that the slope of the regression coefficient is equal to -1. A hyperbolic relationship between β-cell response and IS has, during the last few decades, been described in several species including rodents, dogs and humans (Kahn *et al.*, 1993; Mittelman *et al.*, 2000; Pacini *et al.*, 2001). This relationship has, to the author's knowledge, not previously been elucidated in horses.

For a relationship that fulfills the criterion for a rectangular hyperbola, the product of β-cell response (y, dependent variable) and IS (x, independent variable) is constant for a given degree of glucose tolerance. This constant is called DI. The DI can be considered a measure of pancreas functionality (i.e. a measure of the β-cells' ability to compensate for decreased IS). A decrease in DI is thus an indication of β-cell dysfunction, resulting in inability to compensate for decreased IS (i.e. impaired glucose tolerance and type II diabetes mellitus) (Bergman *et al.*, 2002). During development of IR there is an adequate compensatory increase in the β-cells release of insulin (i.e. normal glucose tolerance) and DI remains constant (Kahn *et al.*, 1993; Bergman *et al.*, 2002).When the β-cells' release of insulin becomes inadequate in relation to IR, glucose intolerance and type II diabetes mellitus develop. This is associated with a decrease in DI represented by deviation of the hyperbolic curve closer towards the origin (Figure 3) (Ahrén & Pacini, 2004; Pacini, 2006).

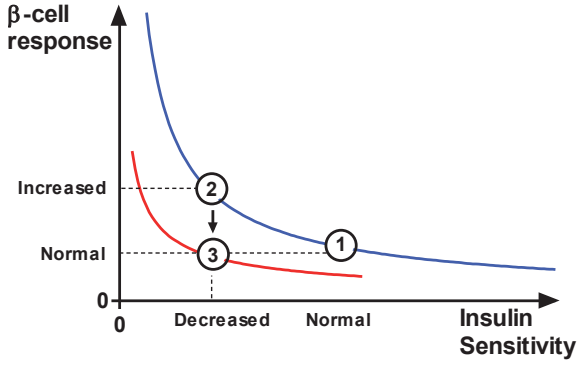


*Figure 3.* Schematic diagram illustrating the characteristics of the rectangular hyperbolic relationship between β-cell response and insulin sensitivity (IS) and the product of the two parameters, the disposition index (DI). State 1: An individual with normal IS and normal β-cell response. State 2: The same individual with an adequate increase in β-cell response in compensation for decreased IS. The DI is unchanged and remains on the same hyperbolic curve. State 3: The same individual that fails to compensate for decreased IS, the β-cell response remains normal whiles the DI is decreased, i.e., a deviation of the hyperbolic curve towards origin.

### 1.7.1 Disposition index

The use of DI is only valid for a non-linear inverse relationship that fulfills the criterion for a rectangular hyperbola. Originally, DI was calculated from the FSIGTT (Bergman *et al.*, 1981; Kahn *et al.*, 1993). This is a convenient way to calculate DI as it requires only one test protocol. Since the introduction of DI in 1981, it has been calculated from IS measured by the EHC, the FSIGTT and the OGTT in conjunction with measurements of β-cell response obtained from the hyperglycemic clamp, the FSIGTT or the OGTT (Kahn *et al.*, 1993; Ahrén & Larsson, 2002; Utzschneider *et al.*, 2006; Retnakaran *et al.*, 2008; Sjaarda *et al.*, 2013).

Not only the measurement of IS and the way in which the β-cell response has been determined, but also the time frame during which the insulin secretion has been evaluated (i.e. early insulin response, total insulin response etc.) has varied between studies. These calculated DIs are therefore not identical and a specific DI is only valid for comparison as long as the same test protocols and analytical methods are used.

# 2 Aims of the thesis

The overall aim for this thesis was to study factors that influence IS in horses and to study the relationship between IS and the postprandial insulin response.

The specific objectives were:

- To evaluate the effect of weight gain induced by a forage-based diet low in NSC and subsequent pasture turnout on IS in moderately IR horses.

- To develop and evaluate a modified OST for determination of the postprandial glucose and insulin response in healthy horses and horses with EMS and to study the response in three different breeds.

- To evaluate the relationship between indices of β-cell response derived from the modified OST and quantitative measures of IS.

- To study the postprandial glucose and insulin response to a meal tolerance test using haylage diets with different content of NSC in horses of two different breeds and to evaluate the relationship between the postprandial insulin response and quantitative measures of IS.

# 3 Comments on materials and methods

This section provides a brief summary on materials and methods used in the separate studies (I – IV) included in this thesis. More detailed descriptions are found in each of the papers.

## 3.1 Horses

All studies were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden. The Standardbreds used in study I – IV were owned by the Department of Clinical Sciences, SLU, Uppsala, Sweden. The healthy Icelandic horses and Gotland ponies used in study III were owned by the Swedish Horse Industry Foundation, Wången, Sweden. All other horses and ponies were privately owned.

All horses included in study I – IV were clinically healthy, had no ongoing episode of laminitis and had negative test results for pituitary pars intermedia dysfunction (plasma ACTH concentrations within the normal reference range for the season). Specific inclusion criteria are reported in each paper. Summarized data for the horses investigated in each of the studies are reported in Table 1. All horses in the EMS group of study II were included in the ID group of study III. The EMS/ID horses were tested during hospitalization after 48 hours of acclimatization to the new environment, whereas all other horses (including four Standardbreds in the ID group of study III) were tested in their home environment.

Table 1. *Summarized data for horses included in study I – IV*

| Study | No | Groups | Breed | Age (mean ± SD) | BCS (scale 1 – 9) | CNS (scale 0 – 5) | Sex | Fitness status |
|---|---|---|---|---|---|---|---|---|
| I | 9 | Moderate IR | Standardbreds | 15.8 ± 3.3 | 5.5 ± 0.6 | 2.4 ± 0.4 | All mares | Sedentary |
| II | 7 | Healthy | Shetland ponies | 3.9 ± 2.7 | 5.6 ± 0.6 | 3.7 ± 0.6 | 4 stallions, 3 mares | Sedentary |
| | 8 | | Iceland horses | 7.0 ± 2.0 | 6.0 ± 0.4 | 2.6 ± 0.4 | 5 geldings, 3 mares | Light exercise |
| | 8 | | Standardbreds | 10.5 ± 4.7 | 5.0 ± 0.5 | 2.0 ± 0.5 | 2 geldings, 6 mares | Sedentary |
| | 20 | EMS* | Mix of pony breeds, riding horses and ponies, Icelandic horses, crossbred horses and ponies. | 13.9 ± 4.6 | 6.9 ± 1.1 | 3.7 ± 0.6 | 5 geldings, 15 mares | Some in light exercise, others sedentary |
| III | 23 | NIR | Standardbreds, Icelandic horses, Swedish warmbloods, Gotland ponies | 9.5 ± 3.9 | 5.3 ± 0.6 | 2.2 ± 0.5 | 12 geldings, 11 mares | Standardbreds sedentary, all others in light to moderate exercise |
| | 26 | ID* | Mix of pony breeds, riding horses and ponies, Standardbreds, Icelandic horses and crossbred horses and ponies. | 14.1 ± 4.3 | 6.7 ± 1.1 | 3.5 ± 0.7 | 5 geldings, 21 mares | Some in light exercise, others sedentary |
| IV | 9 | Healthy | Standardbreds | 14.2 ± 4.7 | 5.4 ± 0.4 | 2.1 ± 0.2 | All mares | Sedentary |
| | 9 | | Icelandic horses | 12.2 ± 4.5 | 5.6 ± 0.7 | 2.4 ± 0.3 | 5 geldings, 4 mares | Light exercise |

SD, standard deviation; BCS, body condition score; CNS, cresty neck score; EMS, equine metabolic syndrome; IR, insulin resistance; NIR, normal insulin regulation; ID, insulin dysregulation. *All EMS horses in study II were included in the ID group of study III. A specification of the breeds included in the EMS/ID groups of study II and III is provided in each paper.

## 3.2 Study designs

Two different quantitative methods to determine IS were used, the EHC and the FSIGTT. Glucose and insulin response to orally administered sugars was evaluated using the modified OST (developed as part of study II) and the meal tolerance test using haylage diets.

Feed was withheld for 12 hours overnight prior to all testing procedures. In humans, an overnight fast prior to both intravenous and oral glucose testing has been recommended (DeFronzo *et al.*, 1979; Stumvoll *et al.*, 2000; Muniyappa *et al.*, 2008). In horses, the time of feed withdrawal has varied widely (i.e. 2 – 13 hours) between studies using dynamic oral glucose tests and intravenous tests for IS (Pratt *et al.*, 2005; Banse & McFarlane, 2014; De Laat *et al.*, 2016; Dunbar *et al.*, 2016; Smith *et al.*, 2016). Time of feed withdrawal will effect gastric retention time after oral administration of sugars. In order to obtain consistent results in the glucose dynamics it is therefore important to empty the stomach prior to oral glucose testing. Recently, a smaller study suggested that a 3-hour period without feed was sufficient, prior to testing with an OST (Bertin *et al.*, 2016). The most optimal time of feed withdrawal prior to testing with oral and intravenous glucose tests needs to be further investigated.

Insertion of intravenous catheters (Intranule, 2.0 x 105 mm, Vygon, Ecouen, France) was performed under local anesthesia (EMLA, AstraZenica AB, Södertälje, Sweden) the night before the different test procedures (i.e. OST, EHC, FSIGTT and meal tolerance test). Two catheters were used for the EHC and the FSIGTT procedures, one for infusion of glucose and insulin and one for blood sampling. For the OST and the meal tolerance test, one single catheter for blood sampling was used. Body weight, general body condition (BCS, scale 1 to 9) (Henneke *et al.*, 1983) and local adiposity (CNS, scale 0 to 5) (Carter *et al.*, 2009a) were assessed in all hoses. The study protocols for study I – IV are briefly described below.

### 3.2.1 Study I

Study I was performed as a longitudinal study lasting for 30 weeks divided into five different periods, P1 to P5. After a maintenance period (P1), weight gain was induced by a continuous increase of energy intake through a diet consisting of haylage (amount adjusted to voluntary intake of each individual horse), supplemented with pelleted lucerne and rapeseed oil (P2). This was followed by a steady-state period (P3), where horses were fed the same diet at an average of 2.5 times their daily maintenance requirement of metabolizable energy (ME)

(NRC, 2007). This feeding period was followed by adaptation to pasture for one week (P4) and subsequently a 4-week period at pasture during the most intensive growing season of pasture grass (P5).

All horses were examined with an EHC before weight gain (end of P1), after weight gain (end of P3) and after four weeks at pasture (end of P5). Fasting (P1 to P5) and postprandial (P5) blood samples were collected at a regular basis to monitor changes in concentrations of plasma insulin and other metabolites. Body weight, BCS and CNS were recorded at the start of the study and regularly during the 30 week long study period.

### 3.2.2 Study II and III

All horses in study II and III were examined with an OST in which Dansukker glykossirap was administered orally at a dose of 0.2 ml/kg BW. Blood samples were collected immediately before (-5 min) and at 30, 60, 90, 120, 150 and 180 min after oral administration of glucose syrup, for subsequent determination of plasma glucose and plasma insulin. To assess the repeatability of the OST in study II, the Icelandic horses and Shetland ponies in the healthy group were examined with the OST twice with 8 days in between. All horses in study III and the EMS group in study II were examined with an EHC the day after the OST.

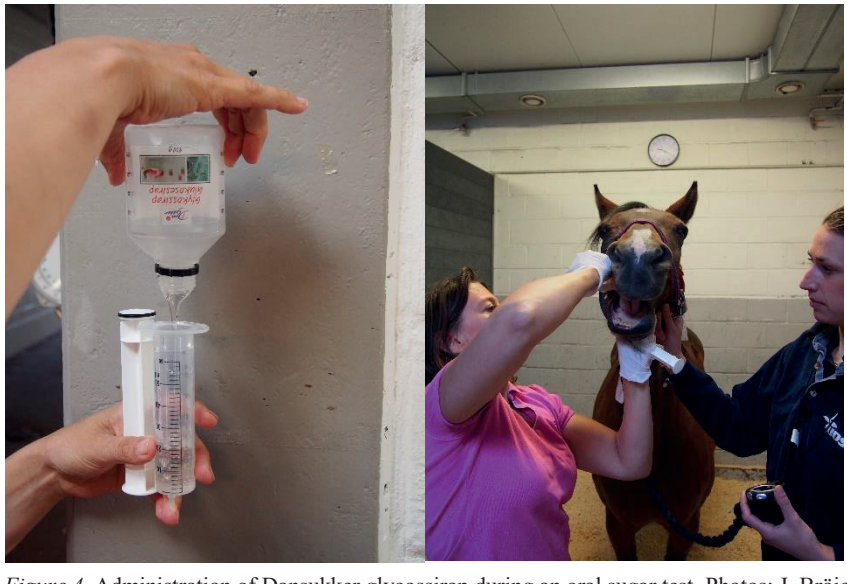

*Figure 4.* Administration of Dansukker glycossirap during an oral sugar test. Photos: J. Bröjer.

### 3.2.3 Study IV

The experiment in study IV was designed as a replicated 3 x 3 Latin square to determine the effects of three different haylage diets with low (LW), medium (MW) and high (HW) water soluble carbohydrate (WSC) content on the postprandial glucose and insulin response. Within breed, horses were assigned to one of three groups with three horses in each group. Each group was fed the three different haylage diets in three periods (P1, P2 and P3). Each period was seven days long. Both breeds were studied in parallel and the order of haylage diets were randomized among groups. The median and range of daily haylage rations during P1 to P3 were 1.2 (1.0 – 1.3) kg DM/100 kg BW, depending on the ME requirement of the individual horse (maintenance only or maintenance and light exercise calculated according to NRC, 2007) and the content of ME in each haylage (LW, MW and HW).

In the morning of the last day of each period, a meal tolerance test was performed in which horses were fed 0.6 kg DM haylage/100 kg BW. This amount was chosen as it represented approximately half of the total daily forage allowance for each horse and was expected to elicit a physiological glucose and insulin response but still allow for two more feedings later during the same day. Blood samples were collected before (-30 and -1 min) feeding and every 30 min post feeding for 5 hours, for subsequent determination of plasma glucose and plasma insulin concentrations. Within two weeks after the last meal tolerance test, all horses were subjected to an FSIGTT.

## 3.3 Quantitative measures of insulin sensitivity

### 3.3.1 Euglycemic hyperinsulinemic clamp (I, II, III)

The EHC technique requires skilled and experienced personnel in order to establish clamps with steady-state situations. Therefore, the same persons performed all 49 clamps. Baseline blood samples were collected after which a continuous rate infusion of glucose (Glucose Fresenius Kabi 500 mg/ml, Fresenius Kabi AB, Uppsala, Sweden) and recombinant human insulin (Humulin Regular, Eli Lilly Sweden AB, Solna, Sweden) was initiated through one of the jugular catheters, using a multi-channel volumetric infusion pump (Colleague, Volumetric infusion pump, Baxter Healthcare SA, Zurich, Switzerland). The infusion rate for insulin was held constant at 3 mIU/kg/min and a variable rate of glucose was infused, to maintain blood glucose concentration at euglycemia (5 mmol/L) during the 3-hour infusion. The glucose infusion rate was adjusted if the concentration deviated by more than 0.2 mmol/L

from euglycemia. Blood samples were obtained every 5 min for immediate analysis of blood glucose concentration using a handheld glucometer (Accu-Check Aviva, Roche Diagnostics Scandinavia AB, Bromma, Sweden) and every 10 min throughout the EHC, for subsequent determination of plasma glucose (10 min intervals) and plasma insulin (20 min intervals) concentrations. Plasma glucose and plasma insulin from the final 60 min of the EHC were used for calculation of:

- Mean glucose disposal rate (M)
- Mean glucose disposal rate per unit of insulin (M/I)
- Metabolic clearance rate for insulin (MCR)

In study III, the M was corrected for non-insulin-mediated glucose uptake (NIMGU) to avoid a confounding factor in the measurement of IS. The relative contribution of NIMGU to total M is small in individuals with normal IS but larger in individuals with IR (Bergman *et al.*, 1989). This situation creates some technical difficulties when it comes to determination of IS in populations with a wide range of IS. In study III, NIMGU accounted for approximately 10% of total M in horses with normal IS, whereas in most IR horses NIMGU accounted for between 55 and 69% of total M. It is therefore important to correct for this error since it is not homogenous.

The NIMGU has been shown to have a linear relationship with blood glucose concentrations in both humans and dogs since it is driven by a mass action effect of glucose (Edelman *et al.*, 1990; Ader *et al.*, 1997). This linearity is constant as long as there is no increase in non-insulin-dependent glucose uptake. Alterations in non-insulin-dependent glucose uptake has been observed in humans with type II diabetes mellitus (Capaldo *et al.*, 1986) and in critically ill patients (Van den Berghe, 2004). Neither of these situations are applicable for the horses used in study III.

The value for NIMGU was obtained by extrapolating data from NIMGU calculated from hyperglycemic clamps in healthy Standardbreds with somatostatin induced insulinopenia (Geor *et al.*, 2010). A weakness is, thus, that NIMGU was calculated rather than measured in the present study. The NIMGU could have been established for the individual horses by performing stepwise hyperglycemic clamps with simultaneous suppression of insulin release from pancreas. This was not possible to achieve, as the cost would have increased significantly and the addition of another clamp would have generated a too labor-intensive study.

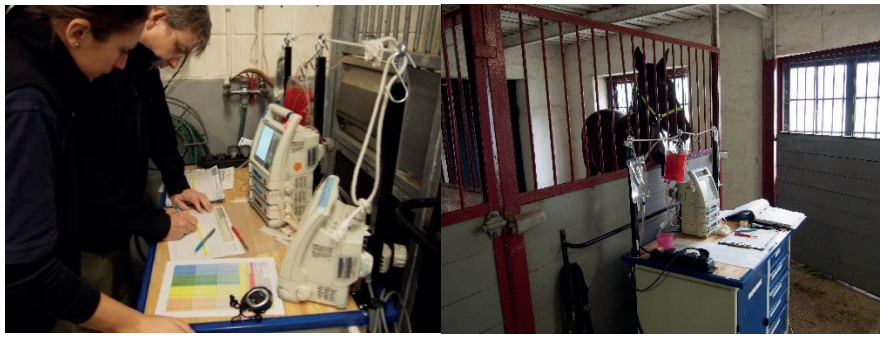

*Figure 5.* Euglycemic hyperinsulinemic clamp procedure. Photos: S. Lindåse., K. Nostell.

### 3.3.2 Frequently sampled intravenous glucose tolerance test (IV)

Baseline blood samples were collected. Thereafter, glucose (Glucose Fresenius Kabi 500 mg/ml, Fresenius Kabi AB, Uppsala, Sweden) at a dose of 300 mg/kg BW was infused intravenously. After 20 min, 20 mIU/kg BW of recombinant human insulin (Humulin Regular, Eli Lilly Sweden AB, Solna, Sweden) diluted into 5 ml of sterile saline (0.9% NaCl) was administered intravenously using the same catheter. Blood samples were collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min in relation to completion of the glucose infusion (corresponding to 0 min), for subsequent analysis of plasma glucose and plasma insulin.

Plasma glucose and plasma insulin data were analyzed using the minimal model method (MinMod Millennium software, version 6.02; University of Pennsylvania, Kennett Square, PA, USA) which provides estimates for:

- Acute insulin response to glucose (AIRg)
- Insulin sensitivity index (Si)
- Glucose effectiveness (Sg)
- Disposition index (DI)

## 3.4 Response variables from the oral sugar test and the meal tolerance test (II, III, IV)

Glucose and insulin response to orally administered sugars was evaluated using the OST (study II, III) and the meal tolerance test with haylage diets (study IV). Postprandial glucose and insulin concentrations were used to calculate different response variables listed in Table 2. Some of the response variables were used as indices to estimate the β-cell response or the IS.

Table 2. *Variables calculated from postprandial plasma glucose and plasma insulin concentrations in response to orally administered sugars*

| Response variable | Test | Study | Index of β-cell response | Index of IS |
|---|---|---|---|---|
| $Peak_{GLU}$ | MTT | IV | | |
| $Peak_{INS}$ | OST, MTT | II, III, IV | X | |
| $T\text{-}Peak_{INS}$ | OST | II, III | | |
| $AUC_{GLU;\ GLU\text{-}60}$* | OST, MTT | II, IV | | |
| $AUC_{INS;\ INS\text{-}60;\ INS\text{-}180}$* | OST, MTT | II, III, IV | X | |
| $AUC_{INS/GLU\text{-}30;\ INS/GLU\text{-}120;\ INS/GLU\text{-}180}$* | OST | III | X | |
| $INS_{INDEX}$ | OST | III | X | |
| $ISI_{COMP}$ | OST | II, III | | X |
| $ISI_{PEAK}$ | OST | III | | X |
| $ISI_{60;\ 90}$ | OST | III | | X |

IS, insulin sensitivity; MTT, meal tolerance test; $Peak_{GLU}$, peak concentration for glucose; $Peak_{INS}$, peak concentration for insulin; $T\text{-}Peak_{INS}$, time (min) to peak concentration for insulin; $AUC_{GLU;\ GLU\text{-}60}$, total or 60 min area under the curve for glucose; $AUC_{INS;\ INS\text{-}60;\ INS\text{-}180}$, total, 60 or 180 min area under the curve for insulin; $AUC_{INS/GLU\text{-}30;\ INS/GLU\text{-}120;\ INS/GLU\text{-}180}$, 30, 60 or 180 min area under the curve for insulin/glucose; $INS_{INDEX}$, insulinogenic-index; $ISI_{COMP}$, composite whole-body insulin sensitivity index; $ISI_{PEAK}$, peak insulin sensitivity index; $ISI_{60;\ 90}$, 60 or 90 min insulin sensitivity index. *Reported as incremental AUC in study III and IV.

## 3.5 Blood sample collection and analysis

Evacuated tubes containing lithium heparin (Vacuette 9 ml, Greiner Bio-One GmbH, Kremsmünster, Austria) were used for blood sampling during testing with the OST, the EHC, the FSIGTT and the meal tolerance test with haylage diets. Sample tubes were instantly placed on ice for 5 min before centrifugation (10 min, 2700 • g). The supernatant was removed and aliquots of plasma were separated, frozen immediately and stored at -80 °C until later analysis of plasma glucose and plasma insulin concentrations.

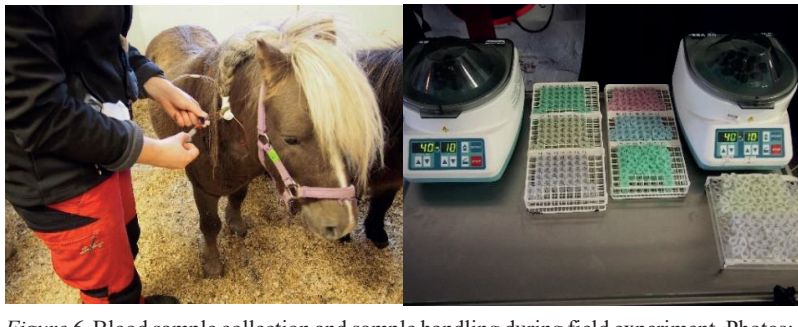

*Figure 6.* Blood sample collection and sample handling during field experiment. Photos: S. Lindåse.

### 3.5.1 Analysis of plasma insulin

Plasma insulin concentrations from EHC and FSIGTT procedures were determined using a commercial human ELISA (Mercodia Insulin ELISA, Mercodia AB, Uppsala, Sweden) and a commercial kit (Mercodia Diabetes Antigen Control (Low, High)/Human, Mercodia AB, Uppsala, Sweden) was used as a control. Endogenous equine plasma insulin concentrations from the OSTs, the meal tolerance tests and the fasting and postprandial blood sampling were determined using a commercial equine-optimized ELISA (Mercodia Equine Insulin ELISA, Mercodia AB, Uppsala, Sweden) and insulin concentrations were controlled with a commercial kit (Mercodia Animal Insulin Control (Low, Medium, High), Mercodia AB, Uppsala, Sweden). All analyses of plasma insulin concentrations were performed in duplicate. Mean intra-assay CVs ranged between 2.2 and 2.7% for equine insulin (equine-optimized ELISA) and between 2.7 and 3.6% for human insulin (human ELISA).

The equine-optimized ELISA has previously been evaluated in our laboratory (Clinical Pathology Laboratory, University Animal Hospital, Uppsala, Sweden) with intra-assay CVs of 2.0 – 6.5% and an inter-assay CV of 10.7% reported (Öberg *et al.*, 2012). Higher intra-assay CVs of 8.4 – 10.6% and an intra-assay CV of 9.0% has been reported for the equine-optimized ELISA in other studies (Tinworth *et al.*, 2011a; Borer-Weir *et al.*, 2012). A recently performed study showed insulin concentration-dependent differences in CVs for the equine optimized ELISA, with higher intra-assay CV (4.6 vs 1.9%) for low (2.3 µIU/mL) insulin concentrations compared to high (109 µIU/mL) insulin concentrations. Also the inter-assay CV was higher (7.3 vs 4.8%) for low insulin concentrations compared to high insulin concentrations (Warnken *et al.*, 2016).

Insulin concentrations analyzed using the equine-optimized ELISA have been reported in the quantitative unit ng/L. Insulin assays optimized for human insulin (e.g. the Mercodia Human Insulin ELISA or Coat-A-Count insulin RIA) typically report insulin concentrations in µIU/mL. During May 2017, the manufacturer (Mercodia AB, Uppsala, Sweden) of the equine optimized insulin ELISA changed the unit of the calibrator curve from ng/L to µIU/mL, to facilitate comparisons between studies using different assays. Data analyzed using the equine-optimized ELISA prior to May 2017 can be converted from ng/L to µIU/mL, using a factor of 1 µIU/mL = 8.7 ng/L. Equine insulin concentrations are reported as ng/L in study I and II and as µIU/mL in study III and IV as well as in this thesis.

### 3.5.2 Analysis of plasma glucose

In study I – III, plasma glucose concentrations were measured enzymatically with an automated clinical chemistry analyzer (YSI 2300 Stat Plus Analyzer, YSI Incorporated, Yellow Spring, Ohio). In study IV, plasma glucose concentrations from the meal tolerance tests were determined with a fluorometric method (KC4 program in BIO-TEK FL600, BIO-TEK instruments INC, Wilrijk, Belgium) (Lowry & Passoneau, 1973), whereas plasma glucose concentrations from the FSIGTT procedures were measured using an automated clinical chemistry analyzer (Architect c4000, Abbott Scandinavia AB Diagnostics, Solna, Sweden). All analyses of plasma glucose were performed in duplicate or triplicate. The mean intra-assay CVs for plasma glucose analyzed using the YSI 2300 range between 0.4 and 0.6%. The mean intra-assay CV for glucose analyzed using the BIO-TEK FL600 was 4.0% and the intra-assay CV for glucose measured with the Architect c4000 was 0.6% based on 10 replicates of the same sample.

## 3.6 Feed sample collection and analysis

In study I, samples of haylage and pelleted lucerne were obtained three times during P1 through P3 and samples of pasture grass were collected every fourth day during P4 and P5. In study IV, samples of haylage were collected at the end of each period (P1, P2 and P3), from the same part of the haylage bale that was fed to the horses during the meal tolerance tests. All feed samples were frozen and stored at -80 °C until chemical analyses were performed.

Analyses of contents of free glucose, free fructose, sucrose, fructans and starch in feed samples were performed using an enzymatic-spectrophotometric method (Udén, 2006). Total NSC content was calculated as the sum of WSC and starch. Total WSC content was calculated as the sum of free glucose, free fructose, sucrose and fructans. The content WSC - fructans (WSC-f) comprises the sum of free glucose, free fructose and sucrose.

## 3.7 Statistical analysis

All data were analyzed in JMP® Pro version 11.0.0, 11.2.0 or 13.0.0 (SAS Institute Inc., Cary, North Carolina, USA). Values where $P < 0.05$ were considered as statistically different in all analyses. All data were analyzed for normality by visual assessment of residual plots prior to analysis. If residuals were not normally distributed, data were either presented as median and interquartile range (study III) or log-transformed. Log-transformed data were either expressed as the geometric mean ± 95% CI on the original scale after back

transformation (study I, II and IV) or presented as log-transformed data in scatter plots (study III and IV). All other data were presented as least square means ± SEM (study IV) or mean ± SD (study I – IV) and range (study II).

Differences in physical characteristics (age, BCS, CNS and BW), OST-derived glucose and insulin variables ($AUC_{GLU}$, $AUC_{INS}$, $ISI_{COMP}$, $Peak_{INS}$ and T-$peak_{INS}$), EHC data (M and M/I) and minimal model variables (Si, Sg, AIRg and DI) were compared between breeds (study II, IV) and between the healthy/NIR and EMS/ID groups (study II and III) by use of an independent t-test, a one-way ANOVA or a nonparametric test (Wilcoxon rank-sum test) as appropriate.

In study I, a one-way ANOVA for repeated measures was used to study differences in BCS, CNS, BW and EHC-derived IS measures over time during the 30 week long experimental period. Because time points were not equidistant, a spacial power covariance structure was used to model the within-horse covariance over time. In study II, a two-way ANOVA for repeated measures was used to study differences in plasma glucose and plasma insulin concentrations over time during the OST, between the healthy and the EMS group. In study IV, a two-way ANOVA with co-variance structure was used to investigate the effect of breed and haylage diet on response variables for glucose and insulin ($AUC_{GLU}$, $AUC_{GLU-60}$, $Peak_{GLU}$, $AUC_{INS}$, $AUC_{INS-60}$ and $Peak_{INS}$) calculated from the meal tolerance tests. A one-way ANOVA was used to identify differences in chemical composition between the different haylage diets (LW, MW and HW). All ANOVAs were run using the mixed model procedure and the Tukey-Kramer post-hoc test was used to identify simple effect differences. Variables were tested for homogeneity of variances by use of the Levene's-test.

In study II, the repeatability of the OST was determined using mean CVs, intraclass correlation coefficients (ICCs) and repeatability coefficients (RCs) calculated for the glucose and insulin response variables ($AUC_{GLU}$, $AUC_{INS}$, $Peak_{INS}$ $ISI_{COMP}$, T-$Peak_{INS}$). Paired t-tests were used to compare mean values for the glucose and insulin variables obtained from the repeated OSTs. In this thesis, mean CVs were calculated for glucose and insulin concentrations for all individual time points during the OST. Bland-Altman analyses (Bland & Altman, 2010) were performed for determination of the mean differences between repeated tests and the RCs for plasma glucose and insulin concentrations for all individual sampling time points during the OST. The differences between repeated tests were checked for normal distribution using histogram plots. The RC was calculated as 2 times the SD of the differences of the repeated glucose and insulin measurements (Bland & Altman, 2010). The differences between repeated tests (measurement error) for insulin

concentrations at 60 and 90 min sampling were plotted against the mean value (estimates the true value) for each subject.

In study III, simple linear regression analyses were used to study the correlation between indices of IS derived from the OST ($ISI_{COMP}$, $ISI_{PEAK}$, $ISI_{60}$ and $ISI_{90}$) and quantitative measures of IS (M and M/I) derived from the EHC. Data were log-transformed prior to analysis to correct for skewed distribution of residuals. Simple linear regressions were also used in study IV, to evaluate the relationship between response variables for insulin ($Peak_{INS}$, $AUC_{INS-60}$ and $AUC_{INS}$) obtained from the meal tolerance tests and quantitative measurements of IS derived from the FSIGTT. Data were log-transformed prior to analysis in order to test if a negative linear relationships was evident. In study III, a variant of linear regressions was used to study the relationship between OST-derived indices of β-cell response ($Peak_{INS}$, $INS_{INDEX}$, $AUC_{INS-180}$ $AUC_{INS/GLU-30}$, $AUC_{INS/GLU-120}$ and $AUC_{INS/GLU-180}$) and EHC-derived IS measures (M and M/I). These regressions were analyzed using the fit orthogonal command in the bivariate platform because this model adjusts for measurement errors in both the y (dependent: β-cell response) and x (independent: IS) variables. Data were log-transformed prior to analysis in order to test if a rectangular hyperbolic relationship was evident.

# 4 Main results

This section summarizes the main results from the separate studies. More detailed descriptions are presented in each of the papers (I – IV).

## 4.1 Effects of obesity and pasture turnout on insulin sensitivity (I)

Mean daily intake of ME increased from 97 to 242% of maintenance requirements from P1 to the end of P2 (Figure 7). During the same time period the mean consumption of WSC-f increased from 48 to 127 g/100 kg BW. During P3, the mean intake of ME was 250% of maintenance requirements and mean consumption of WSC-f was 117 g/100 kg BW during the same time period. The mean WSC and WSC-f content in samples of pasture grass (n = 7) collected during P4 – P5 was 12 and 8% of DM respectively.

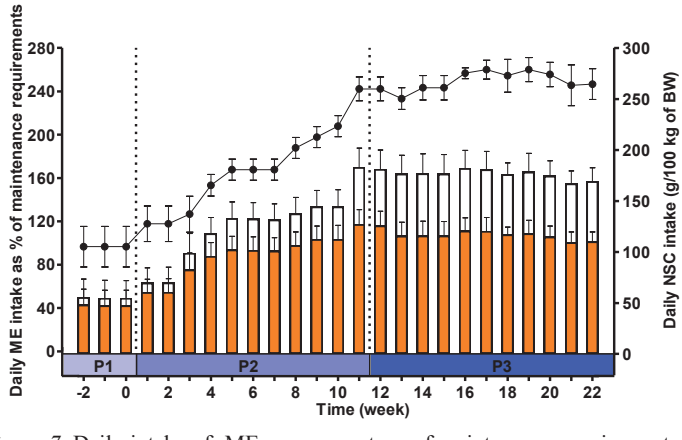


*Figure 7.* Daily intake of: ME as a percentage of maintenance requirements (black circles); NSC (orange + white bars) and WSC-f (orange bars), during maintenance (P1) and weight gain (P2, P3) of study I. Data are mean ± SD for intake on the first day of each week. ME, metabolizable energy; NSC, non-structural carbohydrates; WSC-f, water soluble carbohydrates - fructans.

From the start to the end of weight gain (P1 – P3) horses had a mean increase in BW of 9.2% ($P < 0.001$). The weight gain was accompanied by an on average 0.4 unit ($P = 0.002$) and 1.6 unit ($P < 0.001$) increase in CNS and BCS respectively. There was no further increase ($P > 0.17$) in BW, CNS or BCS during the four week long period at pasture (P4 and P5).

There was no further decrease in M/I and MCR during weight gain, whereas four weeks at pasture increased both M/I and MCR (Figure 8). Thus, the horses' IS were comparable before and after weight gain but improved after the pasture period. The magnitude of the postprandial insulin concentrations at pasture (P5) was comparable with fasting insulin concentrations at the end of weight gain (P3).

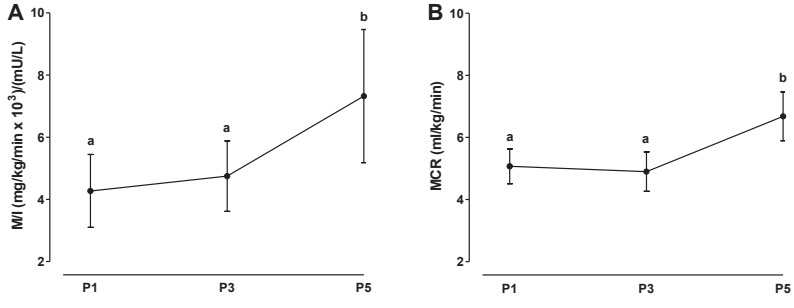


*Figure 8.* Measures of insulin sensitivity: M/I (A) and MCR (B) derived from EHC procedures performed in nine Standardbreds before weight gain (P1), after weight gain (P3) and after four weeks at pasture (P5) during study I. Data are presented as mean ± SD. [a,b]Means with different superscript letters differ at $P < 0.05$. M/I, mean glucose disposal rate per unit of insulin; MCR, metabolic clearance rate for insulin; EHC, euglycemic hyperinsulinemic clamp.

## 4.2 Evaluation of the oral sugar test (II)

Calculated glucose and insulin response variables ($AUC_{GLU}$, $AUC_{INS}$, $Peak_{INS}$, $ISI_{COMP}$ and T-$peak_{INS}$) were not different between repeated tests performed in a group of 15 healthy horses ($P > 0.3$). Mean CVs for calculated response variables and for individual time points during the OST ranged between 6.7 and 10.7% for glucose and between 13.5 and 44.4% for insulin. The lowest and highest mean CV for insulin was found at the 60 and 180 min sampling time point respectively (Table 3). The ICCs were comparable between glucose and insulin response variables ranging between 0.64 and 0.91.

Mean differences for plasma glucose and plasma insulin concentrations between repeated OSTs were all close to 0 (Table 3). The differences were normally distributed and Bland-Altman analyses could be used to calculate RCs for glucose and insulin for individual sampling time points during the OST. The

RCs were larger for insulin compared to glucose for all single time points during the OST (Table 3) as well as for calculated response variables (data reported in paper II, page 60, Table 4). Bland-Altman plots for plasma insulin showed a tendency towards heteroscedasticity at 60 min, whereas a tendency of a negative relationship between the differences and the mean concentration was seen at 90 min (Figure 9).

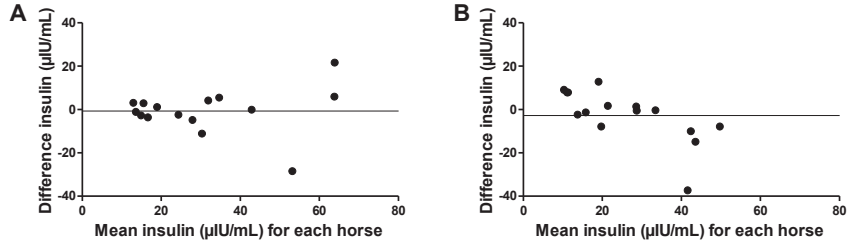


*Figure 9.* Bland-Altman plots showing the paired differences against the mean value for plasma insulin concentrations measured at 60 (A) and 90 (B) min during repeated oral sugar tests in 15 healthy horses in study II. Solid line represents the mean difference between insulin values obtained at repeated testing.

Table 3. *Mean difference and repeatability coefficient (RC) obtained from Bland-Altman analyses and mean coefficient of variation (CV) of plasma glucose and plasma insulin for individual sampling time points during repeated oral sugar tests in 15 healthy horses in study II. Data are presented as mean ± SD*

| | Test 1* | Test 2* | Mean difference* | RC* | CV % |
|---|---|---|---|---|---|
| Glucose - 5 min | 5.5 ± 0.9 | 5.2 ± 0.6 | 0.3 | 1.6 | 6.7 |
| Glucose 30 min | 6.7 ± 1.0 | 6.9 ± 0.8 | -0.3 | 2.4 | 8.7 |
| Glucose 60 min | 7.5 ± 1.0 | 7.5 ± 1.1 | 0.0 | 2.4 | 8.4 |
| Glucose 90 min | 7.1 ± 1.1 | 7.1 ± 1.3 | 0.0 | 2.4 | 10.4 |
| Glucose 120 min | 6.3 ± 1.3 | 6.4 ± 1.1 | 0.0 | 1.6 | 7.8 |
| Glucose 150 min | 5.8 ± 1.2 | 5.5 ± 0.9 | 0.3 | 1.7 | 7.9 |
| Glucose180 min | 5.3 ± 0.9 | 5.1 ± 1.1 | 0.2 | 1.0 | 10.7 |
| Insulin - 5 min | 5.4 ± 3.8 | 5.0 ± 2.6 | 0.4 | 5.9 | 26.2 |
| Insulin 30 min | 28.0 ± 22.1 | 24.4 ± 14.3 | 3.6 | 27.6 | 25.5 |
| Insulin 60 min | 30.7 ± 19.0 | 31.3 ± 17.7 | -0.7 | 21.0 | 13.5 |
| Insulin 90 min | 24.6 ± 10.2 | 27.4 ± 18.1 | -2.8 | 24.5 | 25.5 |
| Insulin 120 min | 17.0 ± 10.6 | 20.0 ± 15.2 | -3.1 | 16.6 | 33.7 |
| Insulin 150 min | 10.7 ± 7.2 | 11.7 ± 8.0 | -1.1 | 11.0 | 27.8 |
| Insulin 180 min | 7.1 ± 4.5 | 8.3 ± 7.2 | -1.1 | 14.9 | 44.4 |

*Data are reported as mmol/L for glucose and µIU/mL for insulin.

There was no difference ($P > 0.2$) between breeds (Shetland ponies, Icelandic horses and Standardbreds) for any of the glucose and insulin response variables ($AUC_{GLU}$, $AUC_{INS}$, $Peak_{INS}$ and $T\text{-}peak_{INS}$) calculated from the OST.

In the comparison between the healthy and the EMS horses, there was a main effect of group during the OST for insulin ($P < 0.0001$) but not for glucose ($P = 0.178$). An interaction between group (healthy vs EMS) and time was identified for both glucose ($P = 0.003$) and insulin ($P < 0.0001$). There was a large variation in the magnitude and shape of the insulin response in the EMS horses whereas the healthy horses had a more homogeneous response. There was a large overlap in glucose response between the healthy and the EMS group (Figure 10). The EMS group (92 ± 27 min) had a delayed mean time to peak concentration for insulin ($T\text{-}Peak_{INS}$) compared to the healthy group ($P = 0.007$; 69 ± 25 min). Geometric means for postprandial insulin response variables ($Peak_{INS}$ and $AUC_{INS}$) were 6 – 7 times higher ($P < 0.0001$) in the EMS compared to the healthy group.

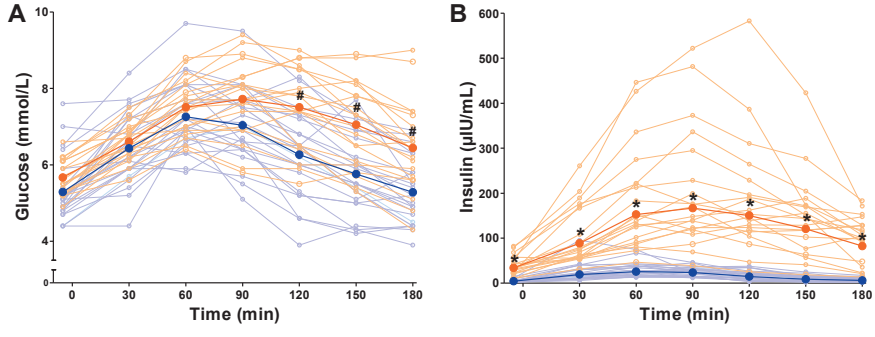


*Figure 10.* Plasma glucose (A) and plasma insulin (B) concentrations measured during an oral sugar test in healthy horses (n = 23) and horses with EMS (n = 20) in study II. Individual horse data (healthy horses, pale blue open circles; EMS horses, pale orange open circles) and mean (glucose) or geometric mean (insulin) for the healthy (blue circles) and the EMS (orange circles) group. *Groups differ at $P < 0.0001$. #Groups differ at $P < 0.004$. EMS, equine metabolic syndrome.

## 4.3 Insulin sensitivity indices from the oral sugar test (II, III)

Linear regression analyses (study III) demonstrated that both the more advanced $ISI_{COMP}$ and the simpler IS indices calculated from one single blood sample during the OST (i.e. $ISI_{PEAK}$, $ISI_{60}$ and $ISI_{90}$) were in good agreement with quantitative measures of IS (M and M/I) from the EHC ($P < 0.0001$; r = 0.81 to 0.86). Selected scatter plots showing the correlation between IS indices from the OST and M/I are shown in Figure 11.

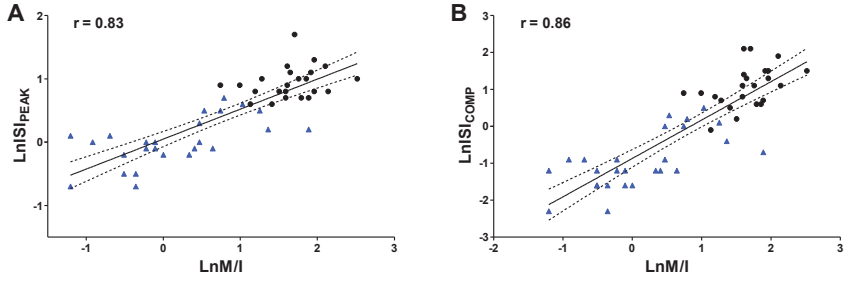


*Figure 11.* Scatter plots showing a linear correlation between log-transformed data for insulin sensitivity indices: $ISI_{PEAK}$ (A) and $ISI_{COMP}$ (B) derived from an OST and M/I derived from an EHC in NIR (black circle) and ID (blue triangle) horses of study III. The solid line is the regression line (line of best fit) and the dashed lines are the 95% CI of the best fit. $ISI_{PEAK}$, peak insulin sensitivity index; $SI_{COMP}$, composite whole-body insulin sensitivity index; OST, oral sugar test; M/I, mean insulin dependent glucose disposal rate per unit of insulin; EHC, euglycemic hyperinsulinemic clamp; NIR, normal insulin regulation; ID, insulin dysregulation.

There was no difference ($P$ = 0.6) in geometric means for $ISI_{COMP}$ between Shetland ponies, Icelandic horses and Standardbreds in study II. However, the geometric mean for $ISI_{COMP}$ was 8 times lower in the EMS group compared to the healthy group ($P < 0.0001$).

## 4.4 Relationship between insulin sensitivity and indices of β-cell response from the oral sugar test (III)

Using indices of β-cell response ($Peak_{INS}$, $INS_{INDEX}$, $AUC_{INS\text{-}180}$, $AUC_{INS/GLU\text{-}30}$, $AUC_{INS/GLU\text{-}120}$ and $AUC_{INS/GLU\text{-}180}$) derived from the OST as dependent variables and quantitative measures of IS (M and M/I) as independent variables in the regression model: Ln (y) = constant + β • Ln (x), demonstrated a negative linear relationship for all 12 comparisons ($P < 0.05$; r = -0.73 to -0.87). This verifies that a non-linear inverse relationship exist between the β-cell response and IS in horses.

The criterion for a rectangular hyperbolic relationship (established if the regression coefficient (β) was equal to -1) was fulfilled for all 6 comparisons between indices of β-cell response against M/I. In contrast, only 3 out of 6 indices of β-cell response ($INS_{INDEX}$, $AUC_{INS/GLU\text{-}30}$ and $AUC_{INS/GLU\text{-}120}$) demonstrated a hyperbolic relationship when IS was determined by the M-value. Selected scatterplots showing a rectangular hyperbolic relationship between indices of β-cell response and M/I are presented in Figure 12.

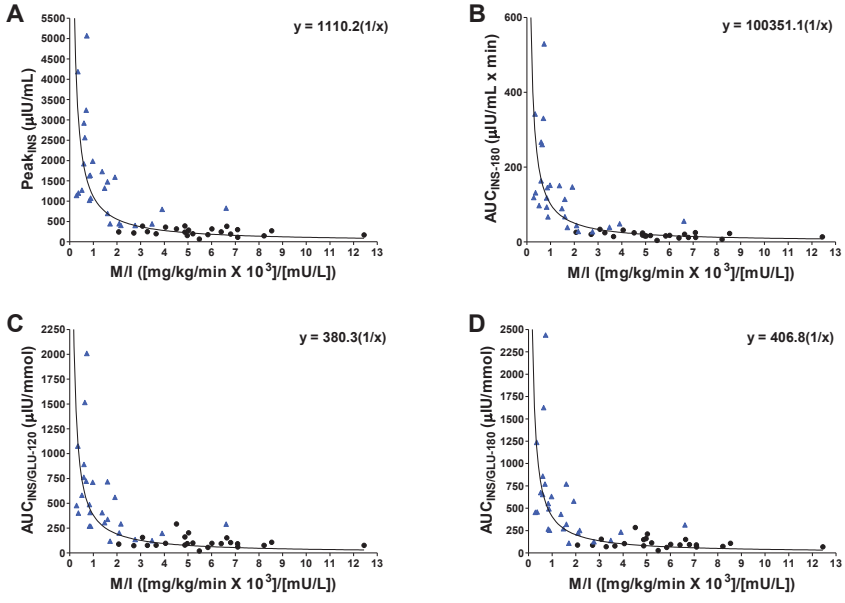


*Figure 12.* Scatter plots of individual horse data (n = 49) illustrating a rectangular hyperbolic relationship between indices of β-cell response: $Peak_{INS}$ (A), $AUC_{INS-180}$ (B), $AUC_{INS/GLU-120}$ (C) and $AUC_{INS/GLU-180}$ (D) derived from an OST and M/I derived from an EHC in NIR (black circle) and ID (blue triangle) horses of study III. A solid line (line of best fit) represents data fitted to a rectangular hyperbolic curve based on the function (y = constant • 1/x), where the constant = disposition index. $Peak_{INS}$, peak concentration of insulin; $AUC_{INS-180}$, area under the curve for insulin at 180 min; $AUC_{INS/GLU-120}$, area under the curve for insulin/glucose at 120 min; $AUC_{INS/GLU-180}$, area under the curve for insulin/glucose at 180 min; M/I, mean insulin dependent glucose disposal rate per unit of insulin; EHC, euglycemic hyperinsulinemic clamp; NIR, normal insulin regulation; ID, insulin dysregulation.

## 4.5 Postprandial glucose and insulin response from the meal tolerance test (IV)

The mean WSC and WSC-f content of DM was 4 and 3% for LW haylage, 13 and 11% for MW haylage and 18 and 11% for HW haylage respectively. The content of WSC-f was comparable between MW and HW haylage ($P = 0.5$) but higher for both MW and HW haylage compared to LW haylage ($P = 0.0001$ for both comparisons). The fructan content was higher in HW haylage compared to both LW and MW haylage ($P = 0.02$ and 0.006 respectively).

All glucose ($AUC_{GLU}$, $AUC_{GLU-60}$, $Peak_{GLU}$) and insulin ($AUCI_{NS}$, $AUCI_{INS-60}$ and $Peak_{INS}$) response variables were higher after a meal of both MW and HW haylage in comparison to LW haylage ($P = 0.0001 – 0.043$) when both breeds were combined. There was no difference between breeds in IS (Si; $P = 0.75$),

but the early postprandial glucose ($AUC_{GLU-60}$) and insulin ($AUC_{INS-60}$) response was higher in Icelandic horses in comparison to Standardbreds ($P$ = 0.004 and 0.003 respectively) when all haylage diets were combined.

## 4.6 Relationship between insulin sensitivity and postprandial insulin response from the meal tolerance test (IV)

Insulin response variables ($Peak_{INS}$, $AUC_{INS}$ and $AUC_{INS-60}$) from the meal tolerance tests as dependent variables and Si from the FSIGTT as independent variable were evaluated in the regression model: Ln (y) = constant + β • Ln (x). This demonstrated a negative linear correlation between the insulin response variables and Si after feeding horses a meal of MW and HW haylage ($P$ < 0.02; r = -0.55 to -0.72). This relationship was, however, not found when the horses were fed LW haylage ($P$ > 0.054; Figure 13). Thus, a non-linear inverse relationship between IS and the postprandial insulin response was only present when haylage with WSC content of ≥ 13% of DM was fed.

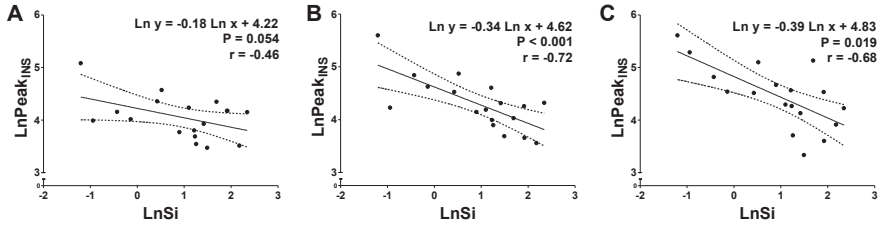


*Figure 13.* Scatter plots showing the correlation between log-transformed data for $Peak_{INS}$ obtained after feeding haylage with 4% (A), 13% (B) and 18% (C) water soluble carbohydrate content of dry matter and Si derived from an FSIGTT in Icelandic horses and Standardbreds (n = 18) of study IV. The solid line is the regression line (line of best fit) and the dashed lines are the 95% confidence interval of the best fit. $Peak_{INS}$, peak concentration of insulin; Si, insulin sensitivity index; FSIGTT, frequently sampled intravenous glucose tolerance test.

# 5 General discussion

## 5.1 Effects of obesity, diet and breed on insulin sensitivity and postprandial insulin response (I, II, IV)

Obesity has been implicated as a contributing factor for development of IR and hyperinsulinemia in several different species including humans, dogs, rabbits and horses (Peiris *et al.*, 1988; Hoffman *et al.*, 2003; Gayet *et al.*, 2004; Frank *et al.*, 2006; Vick *et al.*, 2007; Erdmann *et al.*, 2008; Zhao *et al.*, 2008). During recent years, the effect of diet-induced obesity on IR in horses has been investigated in several studies.

In one study horses were fed a concentrate supplemented diet rich in NSC during 16 weeks, which resulted in a 20% weight gain and a decrease in IS (Carter *et al.*, 2009b). The experimental protocol used in study I was similar to the one used by Carter and coworkers (2009b), but a diet low in NSC and high in fat was used in order to induce obesity. In addition, the horses were already in a state of reduced IS at the start of the study. The 22 week long weight gain period induced a 9.3% increase in BW but there was no effect on the horses' IS. This is in agreement with results of studies on horses fed a diet low in NSC and high in fat for 20 or 32 weeks, where an approximate weight gain of 12 – 17% was observed but no decrease in IS was identified (Quinn *et al.*, 2008; Bamford *et al.*, 2016a; Bamford *et al.*, 2016b). These results suggest that other factors, such as the dietary composition, has a greater impact on alterations in IS than short-term weight gain. However, it is important to emphasize that in the different studies discussed, the degree of weight gain varied and horses were only overweight or obese for a short period of time. It is conceivable that a more chronic state of pronounced obesity might play a role in the pathogeneses of IR and hyperinsulinemia in horses. Moreover, studies have shown that weight reduction in IR horses improves IS (Ungru *et al.*, 2012; McGowan *et al.*, 2013;

Morgan *et al.*, 2016), which verifies a causal association between obesity and IR.

Postprandial hyperinsulinemia and decreased IS has been observed in non-obese horses adapted to diets rich in NSC (Hoffman *et al.*, 2003; Treiber *et al.*, 2005a; Pratt *et al.*, 2006). Thus, dietary management should be considered as a key-stone in the control and prevention of IR and laminitis in horses and ponies. At present, based on clinical experience, a forage diet with < 10 – 12% NSC of DM is recommended for horses and ponies with IR and postprandial hyperinsulinemia (Geor, 2013a). However, sufficient scientific evidence to determine if the upper limit of 10 – 12% NSC of DM is the most optimal cut-off is lacking.

In study IV, we evaluated the glucose and insulin response in clinically healthy horses after intake of haylage diets with different content of WSC. Analysis of the content and components of WSC in haylage showed that MW and HW haylage had a comparable content of WSC-f (glucose, sucrose and fructose), whereas the fructan content was higher in HW compared to MW haylage. Fructans are not degraded by mammalian enzymes (Johnson *et al.*, 2013) and are typically fermented in the hindgut of horses. It has been claimed that some fructans might undergo degradation prior to the large intestine of horses (Coenen *et al.*, 2006) and thereby contribute to the postprandial insulin response (Bailey *et al.*, 2007). Despite a higher fructan content of HW compared to MW, the overall insulin response did not differ between the two diets. This indicates that the influence of fructans on the postprandial insulin response was low in the present study. It is therefore more interesting to study the insulin response in relation to the content of WSC-f.

All three haylages used in study IV caused a postprandial insulin response in the horses and the response was approximately 1.5 – 2 times higher after feeding haylage with high (11% of DM) compared to low (3% of DM) WSC-f content. This is in agreement with results from a previous study (Ringmark & Jansson, 2013) in which the insulin response to haylage with 9% WSC-f of DM was roughly 1.5 times higher compared to haylage with 4% WSC-f of DM. However, in the study (Borgia *et al.*, 2011) that has been used as support of the current arbitrary cut-off limit < 10 – 12% NSC of DM in forage, clinically healthy horses had an almost absent insulin response to hay with 2% WSC-f of DM and the insulin response was almost 5 times higher when the horses were fed hay with 12% WSC-f of DM compared to hay with 2% WSC-f of DM. The large variation in insulin response to forage diets with comparable content of WSC-f indicates that other factors besides the forage content of NCS contribute to the insulin secretion from the β-cells in horses.

One important factor that determines the postprandial insulin response is the horse's prevailing IS. This was demonstrated in study III, where a non-linear inverse relationship between β-cell response and IS was found. To study this relationship further, we investigated the relationship between insulin response variables derived from the meal tolerance tests and quantitative measures of IS in study IV. The postprandial insulin response was inversely related to the horses' IS when MW (13% WSC of DM) or HW (18% WSC of DM) haylage was fed. This relationship was however not found when the horses were fed LW haylage (4% WSC of DM). This demonstrates that the postprandial insulin response after a meal of haylage is affected not only by the content of simple sugars but also depends on the horse's IS. However, the influence of IS on the postprandial insulin response appears to be diminished when the content of WSC in forage is low. Together these results highlight the difficulties of establishing a safe upper cut-off limit for NSC content in forage, applicable for all laminitis prone horses. However, the results indicate that horses with severe IR and highly upregulated β-cell response should be fed a forage diet with lower content of NSC than what is currently recommended. In addition, future recommendations should be based on the content of WSC-f instead of total NSC or WSC, since the postprandial insulin response is more related to the content of simple sugars.

Hyperinsulinemia has been observed in horses and ponies when grazing pasture with high NSC content (Hess *et al.*, 2005; Treiber *et al.*, 2008). In study I, obese horses with reduced IS and a decreased metabolic clearance rate for insulin were examined with an EHC after four weeks of unlimited exposure to pasture grass. Contrary to the expected results, continuous access to pasture did not cause a further decrease in IS, but instead IS was improved, which was accompanied by a proportional increase in the metabolic clearance rate for insulin. Furthermore, the horses did not develop postprandial hyperinsulinemia during the pasture period.

A study (McIntosh, 2007) of grazing Thoroughbred mares revealed a variation in postprandial insulin concentrations and simple indices of IS during the pasture period. Hyperinsulinemia and decreased IS was apparent when the content of WSC was 19% of DM but not when the WSC content was ≤ 10% of DM (McIntosh, 2007). In the present study, the mean WSC content of pasture grass was 12% of DM, which might have been too low to have an effect on IS. The total feed intake for each individual horse during pasture is difficult to predict. However, as the horses did not continue to increase in BW or BCS at pasture, a decrease in nutrient intake compared to the weight gain period is likely. This could possibly explain why a further decrease in IS was not observed, but it is harder to clarify why IS was improved. Improved IS in horses has been reported in response to adaptation to diets rich in NSC

(1.5 – 2.0 g/kg BW) fed to horses once or twice daily (Bamford *et al.*, 2016b; Jacob *et al.*, 2017). Interestingly, in one of these studies (Bamford *et al.*, 2016b) the IS increased in response to the high NSC diet despite a concurrent increase in BW of approximately 12%.

Several factors such as the dose and the chronicity of the stimulus (i.e. feedings /day, duration of high NSC intake) and, possibly more important, a metabolic predisposition for IR, thus likely contribute to whether or not horses develop IR in response to high NSC diets. An inherited predisposition for EMS has been suggested (Treiber *et al.*, 2006b), as certain breeds of horses and ponies more commonly exhibit the EMS phenotype (Frank *et al.*, 2006; Bailey *et al.*, 2008; Bamford *et al.*, 2014). The breed used in study I, Standardbreds, is a breed that does not commonly exhibit the EMS phenotype, thus results might have been different if a breed with a genetic predisposition for IR had been used.

Breed related differences in IS and postprandial insulin responses have been identified in several studies. Higher insulin response to an in-feed OGTT or a NSC rich meal, as well as lower IS, has been found in a group of ponies and Andalusian horses when compared to Standardbreds (Bamford *et al.*, 2014; Bamford *et al.*, 2015; Bamford *et al.*, 2016b). Lower IS and higher postprandial OST insulin response has also been reported for breeds such as Arabian horses and Welsh Mountain ponies when compared to Quarter horses (Manfredi, 2016). When a group of native British ponies were compared to a group of mixed horse breeds, the postprandial insulin response was higher in ponies compared to horses after both an OST and an in-feed OGTT (Smith *et al.*, 2016).

In contrast to these previous studies, no differences in glucose and insulin response variables derived from the OST were found between Shetland ponies, Icelandic horses and Standardbreds in study II. One explanation might be that the OST is not sensitive enough to detect small differences in postprandial insulin response between the different breeds. However, in addition to this, no differences between Icelandic horses and Standardbreds were found for quantitative measures of IS derived from the FISGTT in study IV. A small sample size in combination with a relatively large within-breed variation could explain why no effect of breed was found in study II and IV. There was also a difference in physical activity between the breeds in both studies. As exercise enhances IS in horses (Powell *et al.*, 2002; Pratt *et al.*, 2006; Stewart-Hunt *et al.*, 2006; Morgan *et al.*, 2016), it is possible that the outcome would have been different if the degree of activity had been similar between breeds. Increased physical activity might also have contributed to the improved IS seen in Standardbreds after four weeks at pasture in study I.

Despite similar IS (Si) between the Icelandic horses and Standardbreds in study IV, the early postprandial glucose ($AUC_{GLU-60}$) and insulin ($AUC_{INS-60}$)

response was higher in the Icelandic horses compared to the Standardbreds. This indicates that breed related variations linked to gastrointestinal factors such as incretin effects and glucose absorption might be present. Identification of breed related features in glucose and insulin dynamics is challenging, as other factors such as age, sex, season, diet and exercise might influence the insulin response and IS (Stewart-Hunt *et al.*, 2006; Treiber *et al.*, 2008; Vervuert *et al.*, 2009; Borer-Weir *et al.*, 2013; McCue *et al.*, 2015; Jacob *et al.*, 2017). Thus, it might be necessary to study larger populations of horses under controlled conditions to account for these factors.

## 5.2 Evaluation of the oral sugar test (II, III)

Dynamic tests that involve oral administration of sugars mimic the feeding situation and makes it possible to study postprandial insulin responses under controlled conditions. In 2010, an OST was developed and evaluated in horses in the USA using a commercially available glucose syrup, Karo® light corn syrup (Schuver *et al.*, 2010; Schuver *et al.*, 2014). The Karo® light corn syrup is not available on the Scandinavian market. Our research group therefore decided to develop a modified OST using a Scandinavian commercially available glucose syrup, Dansukker glykossirap (study II).

For the Corn syrup OST, a dose of 0.15 ml Karo® light corn syrup/kg BW is used (Schuver *et al.*, 2014), providing approximately 150 mg saccharides/kg BW. The composition of the saccharides included in the Karo® light corn syrup is withheld by the manufacturer, but it is assumed that the saccharides, other than glucose, have the potential to be degraded to glucose in the small intestine. A dose of 0.2 ml Dansukker glykossirap/kg BW was selected for the modified OST, which provides 216 mg saccharides/kg BW. The Dansukker glykossirap is produced from wheat starch and the composition of glucose, maltose and maltotriose is known. In addition, this glucose syrup contains oligo- and polysaccharides that consist of glucose units. The total dose of saccharides (glucose units) provided to the horses using the modified OST is higher compared to the Corn syrup OST (216 vs 150 mg saccharides). The content of saccharides of both the Corn syrup OST and the modified OST is, however, much lower than the glucose dose used in an OGTT (1 – 1.5 g glucose/kg BW). Recently, a higher dose of Karo® light corn syrup (0.25 ml/kg BW), providing approximately 250 mg saccharides/kg BW, has been used in order to elicit an insulin response in very insulin sensitive horses (Manfredi, 2016; Jacob *et al.*, 2017). This higher dose of Karo® light corn syrup provides a similar dose of glucose units as the modified OST (250 vs 216 mg saccharides). However, the most optimal dose of sugar syrup for the diagnosis of ID still needs to be

established. The administration of sugar syrup becomes less manageable with increasing doses, which also has to be take into consideration if a higher dose should be recommended in the future.

For the Corn syrup OST, peak insulin concentrations generally occurred at 60 – 90 min after syrup administration in both healthy horses and in horses with EMS (Schuver *et al.*, 2010; Schuver *et al.*, 2014). This time interval has been used for sampling in clinical practice since then. In contrast, healthy British native ponies reached their peak insulin concentration already at 30 min post syrup administration (Knowles *et al.*, 2017). In study II, we did not find any breed differences in time to peak insulin concentration during the OST, while the EMS horses had a longer time to peak insulin compared to the healthy horses (92 ± 27 min vs 69 ± 25 min). Plasma insulin concentrations were higher at all time points during the OST in the EMS group compared to the healthy group. The largest difference in plasma insulin concentrations occurred between 60 and 150 min post syrup administration. In agreement with our results, a recent study performed on a larger population of horses subjected to the Corn syrup OST showed that horses and ponies defined as IR based on an FSIGTT, had higher insulin concentrations between 60 and 120 min post syrup administration compared to IS horses (Manfredi, 2016). This suggests that the time frame for single blood sampling during an OST could be extended to include 120 min, which makes the test protocol more time flexible.

An arbitrary cut-off limit for insulin of > 60 µIU/mL for blood sampling at 60 – 90 min has been recommended for diagnosis of ID using the Corn syrup OST (Frank, 2011; Frank & Tadros, 2014). This cut-off value for insulin is reported in µIU/mL, while our insulin results from study II are reported in ng/L. A conversion factor of 10 (1 µIU/mL = 10 ng/L) (Öberg *et al.*, 2012) was used in study II for comparisons between test results. The cut-off insulin concentration of 60 µIU/mL corresponded to 600 ng/L in study II. A lower arbitrary cut-off value for insulin of > 45 µIU/mL for blood sampling at 60 – 90 min has recently been suggested for the Corn syrup OST (Equine Endocrinology Group, 2016) and this cut-off value has been used to define ID in some other studies (Restifo *et al.*, 2016; Frank *et al.*, 2017; Frank & Walsh, 2017). Using the conversion factor of 8.7 (1 µIU/mL = 8.7 ng/L) provided by the manufacturer of the equine-optimized ELISA (Mercodia AB, Uppsala, Sweden) and re-examining the results from study II shows that out of the horses in the healthy group 91% at the 60 min sampling and 100% at the 90 min sampling had plasma insulin concentrations of < 45 µIU/mL. For the EMS horses, 100 and 95% had insulin concentrations of > 45 µIU/mL at 60 and 90 min sampling respectively. The cut-off concentration for insulin of 45 µIU/mL is currently being used in our laboratory (Clinical Pathology Laboratory, University Animal Hospital,

Uppsala, Sweden) for diagnosis of ID using the modified OST. This cut-off value was also used to define horses with ID in study III. An even lower cut-off limit for insulin of approximately 30 µIU/mL using the Corn syrup OST has recently been proposed to be indicative of IR based on results from ROC-curves with the FSIGTT as gold standard method for IR (Manfredi *et al.*, 2016; Manfredi, 2016). Thus, the most optimal cut-off OST insulin concentration for diagnosis of ID is yet to be established.

A higher repeatability (i.e. lower mean CVs and lower RCs) was found for glucose compared to insulin for the individual sampling time points during the OST in study II. Higher repeatability for glucose compared to insulin dynamics of the Corn syrup OST has been found in a previous study reporting mean CVs of 6.4% for $AUC_{GLU}$ and 45.1% for $AUC_{INS}$ (Schuver *et al.*, 2014). In spite of lower variability, the glucose dynamics during an OST has a poor ability to separate healthy horses from horses with ID, visualized by the large overlap in glucose response between the healthy and the EMS group (Figure 10).

The overall repeatability of the insulin dynamics during the OST was considered as moderate based on RCs, ICCs and mean CVs. For insulin concentrations at individual time points during the OST, the best combination of low RC and low mean CV was found at the 60 min sampling. It is not surprising that the insulin dynamics during an OST shows a moderate repeatability as the results are influenced by several physiological factors such as gastric emptying, intestinal glucose absorption and incretin effects (Ferrannini & Mari, 2004; Kronfeld *et al.*, 2005), which all can contribute to the within-horse variability in OST results. The inter- and intra-assay variability for insulin are other factors that can affect the repeatability of the test. Previous studies have reported CVs of 22.6% (Bröjer *et al.*, 2013) and 23.7% (Pratt *et al.*, 2005) for insulin dynamics during repeated testing using the CGIT and the FSIGTT methods respectively. Thus, the repeatability of the OST appears to be comparable to these more specific intravenous methods used for determination of IS.

Mean differences for plasma glucose and plasma insulin concentrations for repeated OSTs derived from Bland-Altman analyses were all close to 0. This indicates that no systemic differences between results from repeated tests were present. Bland-Altman plots for plasma insulin showed a tendency towards heteroscedasticity at 60 min, whereas a tendency of a negative relationship between the differences and the mean concentration was seen at 90 min. Considering the few horses included in this analysis (n = 15) no conclusions of this effect can be drawn, as samples sizes of at least 100 are generally recommended for Bland-Altman analysis (Bland, 2004). The mean values for insulin at 60 and 90 min for the repeated OSTs in healthy horses were 31 and 25 µIU/mL respectively and the RCs for the same time points were 21 and 25

μIU/mL respectively. A healthy horse with insulin concentrations of 31 and 25 μIU/mL at 60 and 90 min sampling of an OST can thus with 95% confidence be expected to have insulin concentrations of 21 and 25 μIU/mL above or below these results at re-testing. In horses with ID, the RCs at 60 and 90 min samplings (calculated from data retrieved from Frank & Walsh, 2017) are higher compared to healthy horses. Due to the higher RC in ID horses, test results should only be interpreted as improved if the insulin concentration during a repeated OST decreases with at least 39 μIU/mL. This variation in absolute insulin concentrations between repeated tests emphasizes that clinicians must be careful when interpreting test results that are close to the cut-off concentration 45 μIU/mL at 60 and 90 min sampling. However, when a binary outcome is used to defined horses as healthy or ID based on insulin concentrations of < 45 or < 60 μIU/mL at 60 – 90 min sampling, the repeatability of the OST is higher (k = 0.65 – 0.80) (Frank & Walsh, 2017; Knowles *et al.*, 2017). Moreover, when the OST was used to compare healthy horses and horses with EMS in study II, insulin concentrations differed between groups for all individual time points during the OST as well as for calculated insulin variables. This indicating that the OST is a valuable test to differentiate normal horses from horses with ID.

It has been suggested that the dynamic oral glucose tests, i.e., the OGTT and the OST, more closely mimic glucose and insulin dynamics during physiological conditions than the EHC or the FSIGTT. The OGTT and the Corn syrup OST have previously been evaluated against different intravenous tests, but only a few studies have used quantitative methods to determine IS (i.e. the FSIGTT or the EHC). A poor agreement as well as a lack of linear relationship between insulin dynamics derived from the OST and quantitative measures of IS obtained from an FSIGTT or an EHC have been reported (Banse & McFarlane, 2014; Dunbar *et al.*, 2016). One explanation for the discrepancy in results might be a lower sensitivity of the OST compared to the quantitative methods. The assumption of a linear relationship between postprandial insulin response data and quantitative measures of IS might be another explanation. In study III, a non-linear inverse relationship was shown between indices of β-cell response derived from the OST and quantitative measures of IS in horses defined as IS to severely IR. Similar results were found in another study in horses with variable IS where insulin data from an in-feed OGTT were compared with Si derived from the FSIGTT (Bamford *et al.*, 2014). This implies that the insulin response derived from oral glucose tests depend on the horse's prevailing IS, even though the tests do not measure IS per se. As a consequence of the non-linear inverse relationship between the β-cell response and IS, large changes in IS for horses with high IS will only result in small changes in postprandial insulin secretion. For horses within the range of normal IS the relationship between the β-cell response and

IS thus might appear as linear, which could explain why a linear relationship has been found between insulin data from an OGTT and quantitative measures of IS in horses with normal IS (Pratt-Phillips *et al.*, 2015). The opposite situation with more pronounced insulin secretion occurs when the horse become IR. This verifies that the OST is not an ideal diagnostic method for identification of horses close to developing IR or with mild IR, i.e., the horses that can be predicted to fall close to the cut-off insulin concentration of 45 μIU/mL during an OST. However, the OST is better suited to detect changes in the postprandial insulin response in horses with marked IR where the insulin secretion is pronounced.

Although the dynamic oral glucose tests does not measure IS per se, different indices based on data from an OGTT have been used both in humans and in horses to estimate IS (Matsuda & DeFronzo, 1999; Gutt *et al.*, 2000; Stumvoll *et al.*, 2000; Pratt-Phillips *et al.*, 2015). These IS indices are mathematical variants of data used for estimating the β-cell response, which by inversion have been transformed to correlate linearly to quantitative measures of IS. A moderate to high linear correlation (r = 0.63 to 0.73) between the most commonly used index in humans, $ISI_{COMP}$ (Matsuda & DeFronzo, 1999), and IS obtained by the EHC have been determined in both humans and horses (Matsuda & DeFronzo, 1999; Pratt-Phillips *et al.*, 2015). In agreement with these previous studies, we found a high correlation between all OST indices ($ISI_{COMP}$, $ISI_{PEAK,}$ $ISI_{60}$ and $ISI_{90}$) and IS measures from the EHC in study III. The $ISI_{COMP}$ could also be used to differ healthy horses from horses with EMS in study II. The results thus verify that OST-derived IS indices can be used to estimate IS in horses. However, as these indices of IS are mathematical variants of data used for estimating the β-cell response, the bias of these data will be propagated to the indices of IS. Thus, the OST-derived indices of IS will not accurately determine IS in horses with mild IR, but becomes valuable for the assessment of improvements or deteriorations in IS in horses with pronounced IR and upregulated β-cell response.

## 5.3 Non-linear inverse relationship between insulin sensitivity and β-cell response (III, IV)

Insulin sensitivity and β-cell response have been shown to be inversely related in humans and dogs (Kahn *et al.*, 1993; Mittelman *et al.*, 2000; Bergman *et al.*, 2002). In study III, we showed that a similar relationship also exists in horses. Moreover, a rectangular hyperbolic relationship was found for 9 of the 12 comparisons between indices of β-cell response derived from the OST and

quantitative measures for IS, which verifies that the OST is a test that can be used to estimate the β-cell response.

In a rectangular hyperbolic relationship, the product of the β-cell response and IS is constant for a given degree of glucose tolerance. This product, DI (Bergman *et al.*, 1981; Kahn *et al.*, 1993; Bergman *et al.*, 2002), can be considered as a measure of pancreas functionality (i.e. a measure of the β-cells' ability to compensate for decreased IS). During development of IR, the β-cells' release of insulin is increased and compensation will be adequate as long as DI remains constant (i.e. compensated IR with normal glucose tolerance). When the insulin release from the β-cells becomes inadequate in relation to IR (decompensated IR), glucose intolerance and type II diabetes mellitus develop, which is associated with a decrease in DI. In horses, type II diabetes mellitus is rare and the β-cells appear to fully compensate for the IR over a long time period. Thus, there are fewer clinical applications for the use of DI in horses compared to humans. Disposition index was originally developed from the minimal modeling of FSIGTT data and is calculated as Si • AIRg (Bergman *et al.*, 1979). During recent years, DI has been reported in several studies of horses subjected to an FSIGTT (Treiber *et al.*, 2005b; Carter *et al.*, 2009b; Bamford *et al.*, 2014; Pratt-Phillips *et al.*, 2015; Dunbar *et al.*, 2016; Jacob *et al.*, 2017). A rectangular hyperbolic relationship between the indices of β-cell response (AIRg) and the measure of IS (Si) derived from the FSIGTT has not yet been demonstrated in horses. Therefore, the use of these DIs as a measure of the pancreas functionally are not justified in the horse.

Even if there is a non-linear inverse relationship between β-cell response and IS, the relationship may not always fulfill the mathematical criterion for a rectangular hyperbolic relationship (Ferrannini & Mari, 2014). Results from study III and IV showed that a non-linear inverse relationship can be described between IS and indices of β-cell response using postprandial insulin data from both the OST and the meal tolerance test with haylage diets. However, only indices of β-cell response derived from the OST correlated hyperbolically to IS. Prior to evaluation of a rectangular hyperbolic relationship between indices of β-cell response and IS, data is log-transformed to fit a linear relationship. A linear regression model is then used that adjusts for measurement errors in both the y (dependent = β-cell response) and x (independent = IS) variables. The criterion for a rectangular hyperbolic relationship is that the regression coefficient (β) is equal to -1.

The error estimates (CVs) for insulin response variables derived from the meal tolerance test using haylage diets were not known prior to the performance of study IV. These error estimates could have been established by repeated meal tolerance tests with the haylage diets used in study IV. This was, however, not

possible due to costs and work load. Therefore, we could not use the model that interprets measurement errors in the dependent and independent variables for data in study IV. Instead, simple linear regressions (accounting for measurements error in the dependent variable only) were used to evaluate the relationship between log-transformed insulin data derived from the meal tolerance test against IS. The data were not tested for a rectangular hyperbolic relationship per se. However, the regression coefficients (β) for MW and HW haylage, when correlated to measures of IS, were -0.39 and -0.34 respectively, which is far from -1. Thus, the meal tolerance test using haylage diets provides less accurate estimates of the β-cell response than the OST. This difference between tests could be related to a larger impact of physiological factors such as gastric emptying and incretin effects when feeding a forage diet, compared to administration of an oral bolus of sugar syrup.

Impaired function of the enteroinsular axis related to defect secretion and action of the incretin hormone glucagon-like peptide 1 (GLP-1) has been shown in humans with type II diabetes mellitus (Nauck *et al.*, 1986; Toft-Nielsen *et al.*, 2001; Vilsbøll *et al.*, 2001). Glucagon-like peptide 1 has been analyzed in horses, but the insulinogenic effect is poorly characterized. Concentrations of GLP-1 have been found to correlate to postprandial insulin concentrations in response to a meal rich in NSC (Bamford *et al.*, 2015) or an in-feed OGTT in horses with ID (De Laat *et al.*, 2016). In contrast, a recently performed study failed to demonstrate differences in GLP-1 concentrations during an OST between healthy horses and horses with EMS, in spite of differences in postprandial insulin response between groups (Chameroy *et al.*, 2016). It has been suggested that postprandial hyperinsulinemia might occur in horses due to alterations in the enteroinsular axis, without presence of IR (Frank & Tadros, 2014; De Laat *et al.*, 2016). In contrast, the data from study III and IV verifies that postprandial insulin response to oral glucose is highly related to the horse's IS. Pronounced hyperinsulinemia after an oral glucose load in horses with normal IS will cause hypoglycemia. Therefore, it is likely that an abnormal postprandial insulin response will be accompanied by an compensatory decrease in IS to prevent the occurrence of hypoglycemia (Corkey, 2012a; Corkey, 2012b). Whether or not IR in horses is the primary requisite or the adaptive response to postprandial hyperinsulinemia is yet to be established.

# 6 Concluding remarks

This thesis contributes to increased knowledge about the pathophysiology, clinical management and recognition of horses with IR and hyperinsulinemia. The following specific conclusions can be drawn from the present work:

- The dietary composition has a larger impact than short-term weight gain on alterations in IS in horses, which suggests that obesity is not a prerequisite for development of IR in horses.

- The OST is an easy and practical test that can be used in field settings for diagnosis of horses with postprandial hyperinsulinemia. The test can also be used to monitor IS in response to clinical interventions in horses with severe IR. No breed related differences in OST responses were detected.

- A rectangular hyperbolic relationship was found between indices of β-cell response derived from the OST and quantitative measures of IS from the EHC. This justifies the use of DI as a measure of pancreas functionality in horses under specific conditions. The results also confirm that the OST primarily is a test that estimates the β-cell response rather than IS.

- The postprandial insulin response depends on both the NSC content in forage and the horse's IS, but the effect of IS is diminished when the forage NSC content is low. This implies that horses with IR and an augmented β-cell response should be fed a forage diet with low content of NSC, in order to decrease the risk for laminitis. Breed related differences in the early postprandial glucose and insulin response were found.

# 7 Future considerations

Through the work of this thesis, we have elucidated aspects of equine IR and postprandial insulin response. However, several questions remain to be answered in future studies. Our results show that short-term obesity per se does not decrease IS in horses in agreement with results from other studies. If a chronic state of obesity has implications in the pathogeneses of IR in horses warrants further investigation. The OST can be used to estimate the β-cell response in horses and appears to be a valuable field test for identification of horses with ID. However, further refinements of the OST must be considered including assessment of optimal dose of glucose syrup, optimal diagnostic cut-off concentration as well as breed related characteristics in the response. Future research on diagnostic methods for ID in horses should be aimed at identify methods that recognize individuals that are close to developing IR and hyperinsulinemia better than the current OST. It is also necessary to investigate if a rectangular hyperbolic relationship between the FSIGTT derived AIRg and Si can be found in horses, to justify the use of DI derived from the FSIGTT as a measure of pancreas functionality.

There are still several questions to be answered on the pathogenesis of equine hyperinsulinemia and IR, including the role of gastrointestinal factors such as alterations in the intestinal glucose absorption and incretin effects. To elucidate the potential role of incretin hormones in the pathogenesis of ID in horses, evaluation of equine specific assays as well as measurements of the incretin effect are warranted. Increased knowledge about these underlying mechanisms of ID in horses is necessary to decide which diagnostic methods most accurately identify these horses. Thus, if the primary deficiency is related to an altered incretin effect, the OST, a test that estimates the β-cell response, is perhaps not the most ideal field test for identification of laminitis-prone individuals. Possibly, other oral glucose tests that more efficiently stimulate the incretin effect, such as meal tolerance tests, could prove more beneficial.

The EMS-phenotype is more frequently recognized in certain breeds of horses and ponies and several previously performed studies have identified breed related differences in insulin dynamics. However, in the presence of confounding innate (e.g. age) and environmental factors (e.g. diet and exercises), it appears that studies on larger populations of horses evaluated under controlled conditions are necessary to enable identification of breed related metabolic features in insulin dynamics. One final interesting aspect that requires further consideration is that horses for some reason appear to be protected from β-cell exhaustion/dysfunction, as they seldom progress from compensated to decompensated IR. This is in contrast to humans and cats that commonly suffer from decompensated IR, impaired glucose tolerance and type II diabetes mellitus. Studies investigating the function of the pancreas in different breeds and species could possibly increase the understanding of IR and β-cell dysfunction in a larger context.

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# Populärvetenskaplig sammanfattning

Fång är ett tillstånd som drabbar hästens hov och som i många fall leder till bestående smärta, hälta och i värsta fall avlivning. Fång kan uppstå till följd av många olika sjukdomar hos häst men en av de vanligaste orsakerna är en obalans i hästens insulinreglering, nämligen insulinresistens och hyperinsulinemi. Insulin är ett hormon som reglerar blodsockret efter intag av föda. Vid normal insulinkänslighet stimuleras kroppens celler att ta upp glukos och blodsockret förblir därmed stabilt efter födointag. Vid insulinresistens svarar inte kroppens celler normalt på det insulin som utsöndras efter födointag. För att förmå cellerna att ta upp glukosen från blodet kompenseras den nedsatta insulinkänsligheten av en ökad utsöndring av insulin till blodet. Detta skapar ett tillstånd av onormalt höga insulinkoncentrationer i blodet, kallat hyperinsulinemi. Genom att i studier tillföra insulin i höga koncentrationer till hästens blod har man inducerat fång hos tidigare friska individer. Detta visar att det finns en koppling mellan hyperinsulinemi och utveckling av fång. Metabolt syndrom hos häst, även kallat ekvint metabolt syndrom (EMS), är ett samlingsbegrepp som används för att beskriva hästar med en ökad risk för att utveckla fång. Både insulinresistens och hyperinsulinemi liksom fetma har beskrivits som huvudkomponenter i sjukdomskomplexet EMS. Man har under årens lopp noterat att ponnyraser så som russ och shetlandsponnyer samt ursprungliga hästraser så som islandshästar, oftare drabbas av EMS jämfört med t.ex. trav- och galopphästar. Det finns därför teorier om en genetisk bakgrundsorsak till EMS. I dagsläget är kunskapen begränsad om relationen mellan de olika komponenterna som ingår i EMS.

Det övergripande målet med denna avhandling var undersöka faktorer som påverkar hästens insulinkänslighet samt att undersöka relationen mellan insulinresistens och hyperinsulinemi. Ett ytterligare mål var att utveckla ett enkelt diagnostiskt test för EMS.

Fetma är ett vanligt förekommande problem hos hästar över hela världen och antalet feta hästar tycks öka i takt med att välfärdssamhället breder ut sig. Hos människa har man visat att fetma är starkt bidragande till utvecklande av

insulinresistens och typ II diabetes mellitus. Hos häst har man noterat att de individer som lätt drabbas av insulinresistens och fång i stor utsträckning även är överviktiga eller feta. Genom att banta dessa individer har man visat att insulinresistensen minskar, dvs. insulinkänsligheten förbättras. Således finns det vetenskapliga bevis för att fetma är kopplat till insulinresistens även hos häst, men om fetma är grundorsaken till att hästar utvecklar insulinresistens är inte känt. Att utfodra hästar med en diet som innehåller stora mängder socker och stärkelse kan leda till insulinresistens och hyperinsulinemi. Höga nivåer av socker och stärkelse finns framförallt i olika typer av kraftfoder. Vi studerade hur viktuppgång påverkar insulinkänsligheten hos häst genom att utfodra en diet med lågt innehåll av socker, bestående av ett vallfoder (hösilage), lusernpellets och olja. Denna diet utfodrades i mängder som motsvarade 2,5 gånger hästarnas näringsbehov. Målet var att undersöka effekten av fetma, utan en samtidig påverkan av en diet med högt socker- och stärkelseinnehåll. Alla hästar i studien ökade med ca 9% i vikt under experimentet utan att insulinkänsligheten försämrades.

Att mäta graden av insulinresistens hos häst är komplicerat och det krävs avancerad utrustning, mycket tid och vältränad personal. Möjligheterna att diagnostisera hästar med insulinresistens och en ökad risk för fång i fält har därför historiskt sett varit begränsade. För att få en indikation på om hästen är insulinresistent har man tidigare använt sig av enkla faste-prover (blodprov som tas innan morgonutfodring) för mätning av glukos- och insulinkoncentrationer. Informationen man erhåller från ett sådant prov är dock mycket begränsad och det finns risk för att insulinresistens och hyperinsulinemi hos individen missas. För att bättre kunna identifiera individer med risk för fång på grund av nedsatt insulinkänslighet skapade vi ett test där man ger en sockersirap i hästens mun, följt av blodprovstagning, kallat oralt sockertest (OST). Ett OST härmar en utfodringssituation och ger alltså information om huruvida hästen får hyperinsulinemi efter intag av föda. Djurägaren ger själv hästen sockersirap i munnen på morgonen efter fasta under natten, vartefter veterinären kommer och tar ett blodprov som analyseras för glukos- och insulinkoncentrationer. Denna metod var redan utvecklad i USA men den sockersirap man använder där går inte köpa i Sverige. Den sockersirap vi valde till vårt test (Dansukker glykossirap) finns att köpa i de flesta mataffärer. Genom att jämföra resultat från en OST mellan friska hästar och hästar med EMS kunde vi konstatera att denna enkla metod kan användas för att särskilja friska hästar från hästar med insulinresistens och hyperinsulinemi.

För att öka förståelsen för relationen mellan insulinresistens och hyperinsulinemi hos häst jämförde vi insulinkoncentrationer från OSTn med en avancerad diagnostisk mätmetod för insulinresistens. Vi kunde genom denna

studie bekräfta att insulinresistens och hyperinsulinemi är beroende av varandra, och att graden av insulinresistens kommer vara avgörande för om hästen får hyperinsulinemi efter utfodring. Detta innebär att en häst som har kraftig insulinresistens också kommer ha en kraftig hyperinsulinemi, men förhållandet är inte linjärt. Om man hos denna individ kan uppnå endast en liten förbättring av insulinkänsligheten (t.ex. genom dietåtgärder) så medför detta en markant sänkning av hyperinsulinemin. Detta minskar således risken för att hästen ska drabbas av fång eftersom fång uppstår till följd av hyperinsulinemi.

Som tidigare diskuterats är insulinresistens och hyperinsulinemi kopplat till intag av fodermedel med högt innehåll av socker och stärkelse. Genom att utfodra en vallfoderdiet utan tillägg av socker- och stärkelserika kraftfoder minskar man därmed hästens totala sockerintag. Dock innehåller även vallfoder socker, och det finns i dagsläget begränsad information om hur sockerinnehållet i vallfoder påverkar hästens insulinreglering. Den nuvarande rekommendationen är att utfodra hästar med risk för hyperinsulinemi och fång ett vallfoder med maximalt 10 – 12% socker och stärkelse i torrsubstansen (ts). Vallfoder producerade i Sverige innehåller inte stärkelse, varför gränsen för svenska förhållanden motsvara 10 – 12% socker (mer specifikt kallat WSC) i ts. Vi undersökte insulinkoncentrationerna hos friska hästar efter utfodring med tre olika hösilage med lågt (4%), medel (13%) och högt (18%) innehåll av WSC. Vi undersökte även hästarnas insulinkänslighet med en avancerad diagnostisk mätmetod. Resultaten visade att insulinkoncentrationerna efter utfodring med hösilage med medel eller högt sockerinnehåll var högre jämfört med när hösilage med lågt sockerinnehåll utfodrades. Även hästens insulinkänslighet var betydande för hur hög insulinkoncentrationen var efter utfodring med hösilage med medel och högt sockerinnehåll. Denna effekt kunde dock inte ses när hösilage med lågt sockerinnehåll utfodrades. Detta innebär att om man utfodrar en redan insulinresistent häst med ett vallfoder med lågt sockerinnehåll, så minskar risken för att hyperinsulinemi och fång ska utvecklas.

Sammanfattningsvis har detta projekt ökat kunskapen om relationen mellan fetma, utfodring, insulinresistens och hyperinsulinemi hos häst, samt skapat ett diagnostiskt test som kan identifiera individer med EMS under fältmässiga förhållanden. Resultaten indikerar att fetma inte är den primära orsaken till att hästar utvecklar insulinresistens, samt att insulinresistens är avgörande för om hyperinsulinemi uppstår efter utfodring. För insulinresistenta hästar är det en viktig åtgärd att ha en låg halt av socker (och stärkelse) i foderstaten för att minska risken för hyperinsulinemi och därmed även för fång.

# Acknowledgements

The work included in this thesis was performed at the Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, SLU. Financial support was generously provided by the research platform **Future Animal Health and Welfare program (FAHW)**. Additional financial support was provided by the **Swedish-Norwegian Foundation for Equine Research** as well as by scholarships provided by the **Faculty of Veterinary Medicine and Animal Science**, **Djurvännernas Förening i Stockholm** and **Michael Forsgrens stiftelse**.

I am truly grateful to all of the **horse owners** and to all of my **helpful and talented colleagues** that have contributed to the completion of this thesis. I am also grateful to **my family and my friends** that have supported me during these years of hard work. I would especially like to express my gratitude to:

**Johan Bröjer**, main supervisor, for introducing me into the interesting field of equine endocrinology. Your great experience within this research topic has been invaluable to me and your thoroughness in research and in the writing process has encouraged me to perform my work with attention and accuracy. I appreciate all the interesting and challenging scientific discussions as well as the fun times we have had together during the years. Your imitations of Carl Bildt and other interesting personalities really made it fun to work, even at 4 AM! Besides being funny, you are also one of the most caring persons I have ever met. It has truly been a privilege to have you as my coach during these years Johan.

**Katarina Nostell**, co-supervisor, for your engagement and participation in my project and for the valuable manuscript feed-back. I admire your positive energy, your straight forward approach and your ability to act and make decisions. I also appreciate your concern for me, as a colleague and as a friend. I have always felt that I can talk to you about things troubling me. I am grateful for all the fun times

we have had together during the different research projects and I really hope this is just the beginning!

**Cecilia Müller**, co-supervisor, for genuinely supporting my work and for patiently teaching me about equine nutrition. Thank you for all the interesting interdisciplinary discussions we have had and for the valuable manuscript feedback. I appreciate how you always find time for me whenever I need to discuss problems that might have arisen.

**Marianne Jensen-Waern**, co-supervisor, for generously sharing your great knowledge within the field of endocrinology. Thank you for always encouraging me to think "big" and outside the box and for your concern for me and for my coming career.

**Anna-Stina Persson, Jessica Hedenskog, Ida Askerfelt, Anna Svensson** and **Malin Zahn**, for organizing and coordinating the clinical trials performed in the field and for excellent technical assistance.

**Ulf Hedenström, Malin Connysson** and **Emelie Granlycke**, for your kind help during the study performed at the Swedish Horse Industry Foundation, Wången.

**Jesper Ordell**, for your assistance and kind contribution of the forage used in study IV.

**Jenny Hedenby, Petra Johansson, Moa Berggren, Elin Hedin, Ellen Winqvist, Camilla Anger, Mimmi Törnqvist, Hanna Edberg** and **Mikaela Mårtensson**, former veterinary students, for your participation in the different projects included in this thesis. It would not have been possible to complete all of these clinical experiments without your help. I am so grateful to all of you.

**IKEA, Uppsala**, for the generous donation of IKEA bags used for packing of the horses' individual haylage rations during study IV.

**Caroline Bröjer**, married to Johan Bröjer, for your patience with all the work outside office hours, it sure has been a lot of them during these years. I am also enormously grateful for your help with the proof-reading of my articles, you are fantastic.

My veterinary colleagues at the Department of Clinical Sciences, the heroes teaching veterinary students in equine medicine and surgery, **Lena Ström, Ove**

**Wattle, Miia Riihimäki, John Pringle, Pia Haubro Andersen, Marie Rhodin** and **Anna Sjöblom**. It has been a pleasure working with you.

All my **friends and colleagues at the horse clinic, UDS**. I could not risk to forget mentioning anyone by name, so I just leave it there. I have had so much fun working with you, during the hectic summers and late nights dealing with acute cases after hours. You are simply the best!

KV-stall, **Mari Wallbring, Carola Jansson** and **Dennis Larsson**, for being so helpful and cheerful during my clinical experiments at old KC and new VHC.

KV-lab, **Anna Svensson** and **Annlouise Jansson**, for patiently helping me in the lab and for sharing your valuable knowledge. Also thank you for your help with my never ending glucose and insulin analyses.

**Susanne Pettersson, Anette Forsberg, Annika Nyström, Zelal Everbring, Elinora Johansson** and **Mikael Rosenius** at the administrative division at the Department of Clinical Sciences, for all your help concerning administrative matters.

Our statistician, **Claudia von Brömssen**, for your excellent and professional help with the statistical models used in study III and IV.

Fellow and former PhD-students, **Ida Hallberg, Anna Malmsten, Denise Laskowski, Theo Ntallris, Karin Olofsson, Maja Wiklund, Emeli Torsson, Frida Martin, Emma Persson Sjödin, Katrina Ask** and many more, for interesting discussions during courses, clinical work and coffee breaks.

My wonderful former and present colleagues in "fikagänget", **Malin Hagberg Gustavsson, Jenny Larsson, Annette Backhans, Hanna Bremer, Lena Pelander, Elin Manell, Axel Sannö, Frida Karlsson, Anna Werinder, Helena Pettersson, Daniel Bergman, Anna Hillström, Emma Strage** and **Ulrika Falkenö**. Thank you for creating a familiar environment at VHC and thank you for all the fascinating discussions on different subjects of life. You make my day, every day.

My roomie, **Malin Öhlund**. Five and a half years together, time flies! I appreciate all the interesting discussions we have had on our common research topic. We have learned a lot from each other during these years and it has truly been a pleasure to undertake this education together with you. Besides this, I

highly value our friendship and your kind support during times when things have been tough. I will miss you so much Mulli!

My dearest friends, colleagues and heroes, **Josefin Söder** and **Ellinor Spörndly-Nees**, for valuable help during my clinical experiments but foremost for taking care of me, for the "go-kaffe", for encouraging me and for making me happy. These years would definitely not have been the same without you.

Wonderful **Elin Hernlund**, for being so cheerful and supporting and for constantly looking at life from the bright side.

My beloved friends from Vet school, **Kerstin Anagrius, Clara Atterby, Lisa Lindström, Emilia Wangel, Anna-Jennie Lyngsmark, Pia Haffling, Mona HansErs, Regina Lindberg, Sandra Candefjord, Karin Lannér, Caroline Olofsson, Sofie Ellström, Cecilia Björkehag, Isabell Björkman, Hanna Ralfe, Rebecka Nyhlén, Emma Tejler, Madeleine Järvinen, Camilla Ahrenbring, Tuva Vamborg, Louise Björnheden, Matilda Widman, Ulrika Jonsson, Helena Gustafsson, Marcus Abrahmsén** and **Thomas Englund**, for all the fun times we have had together during the past 11 years and for great friendship! Especially thank you to **Kerstin, Clara, Emilia, Lisa** and **Anna-Jennie** (over the phone), for spending time with me during dinners and dog walks and for supporting me during my PhD studies.

My hard working and constantly busy friend, **Marie Patriksson**, for being an amazing person. I am so grateful to have a friend like you in my life.

**Malin** and **Adrian Adermon**, my crazy non-veterinarian friends and "extra" family, for taking care of me, Gustav, Tage and the dogs and for being the most incredible persons. Thank you for your complete lack of knowledge about horses and veterinary medicine, it means a lot to me!

**Björn Averhed** and **Lisa Carlegrim**, family and close friends, for all your help with life in general and for spending a lot time with us and Tage. Also thank you to the rest of the "Averhed-clan", for welcoming me into the family.

My childhood friends, **Anna Lindblad, Linda Hedberg Wallenstein** and **Maria Martinelle**, for all the fun we have had growing up together. I am so happy for us keeping in contact, even though we all are busy dealing with the puzzle of life.

**Mamma** and **Pappa**, for your everyday support during my life until now. You are the most amazing persons and I am horned to be your daughter. Thank you for loving me unconditionally, nothing would have been possible without you.

My sister **Malin** with family, for being a huge and important part of my life. We always have so much fun together and we share several charming and some (few!) not so charming characteristics. Thank you for letting me be part of Stellas and Viggos lives.

My sister **Ulrica** with family, for your care about me and my family. I so much appreciate how close we have become during the resent years. Thank you for letting be me a part of Oscars and Davids lives.

My brother **Hannes**, "Lillis", for being who you are.

Last but most importantly, **Gustav** and **Tage**, for making me smile every day and for making me truly happy. Thank you for reminding me about the things that really matters in life. I love you both to bits.